Figure 1

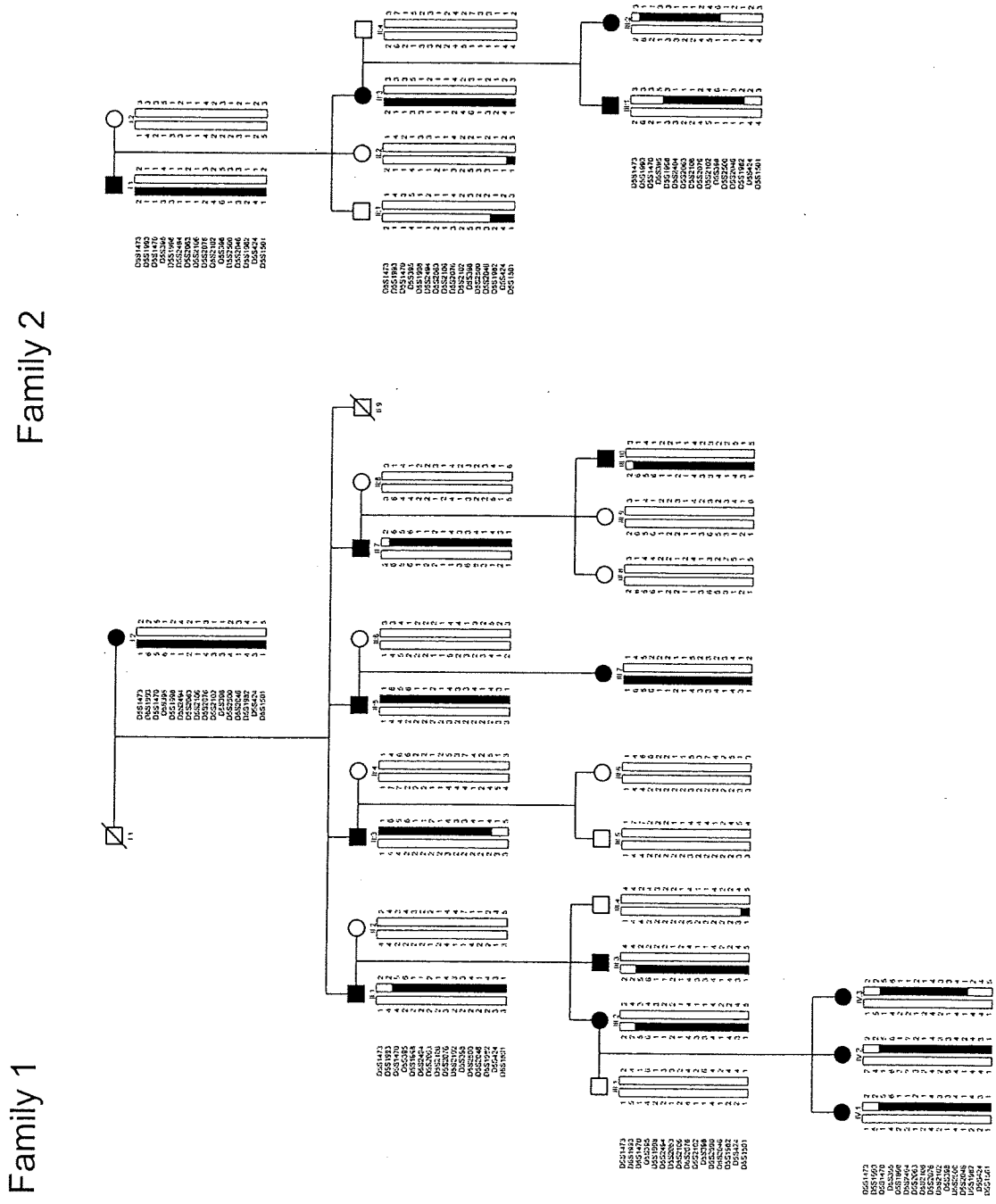


Figure 2

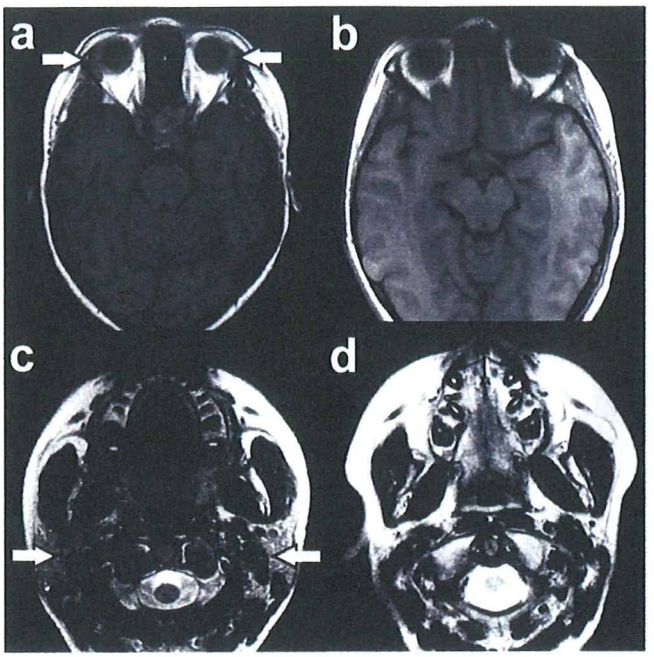


Figure 3

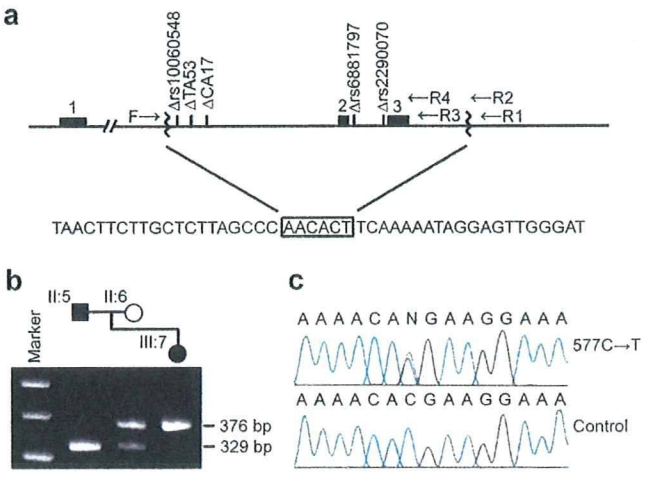
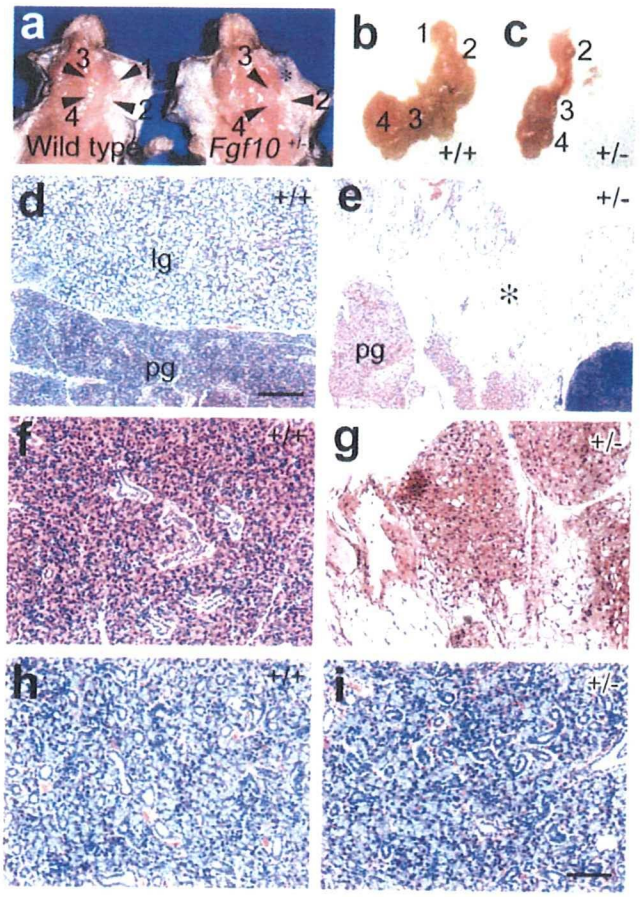
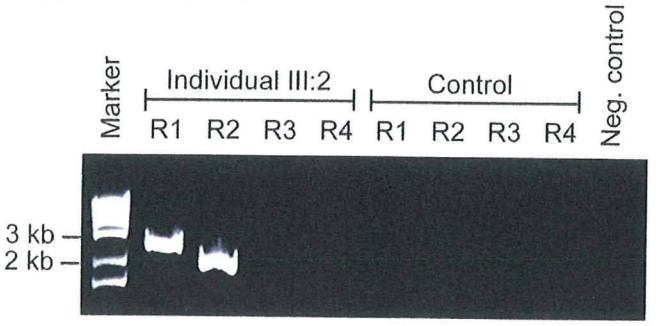


Figure 4



Supplementary Figure 1



Development of Autoimmunity against Transcriptionally Unrepressed Target Antigen in the Thymus of Aire-Deficient Mice

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Running title: **Autoimmunity Against Unrepressed Target Ag Without Aire**

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*Abbreviations used in this paper: AIRE, autoimmune regulator; mTEC, medullary thymic epithelial cell; PHD, plant homeodomain; HEL, hen egg lysozyme; 3d-Tx mice, mice thymectomized 3 days after birth; SS, Sjögren's syndrome; Treg, immunoregulatory T cell; NIK, NF-κB-inducing kinase.

Total character counts: 48,123

Abstract

Autoimmune regulator (AIRE) gene mutation is responsible for the development of organ-specific autoimmune disease with monogenic autosomal recessive inheritance. Although Aire has been considered to regulate the elimination of autoreactive T cells through transcriptional control of tissue-specific Ags in thymic epithelial cells, other mechanisms of AIRE-dependent tolerance remain to be investigated. We have established Aire-deficient mice, and examined the mechanisms underlying the breakdown of self-tolerance. The production and/or function of immunoregulatory T cells were retained in the Aire-deficient mice. The mice developed Sjögren's syndrome-like pathologic changes in the exocrine organs, and this was associated with autoimmunity against a ubiquitous protein, α-fodrin. Remarkably, transcriptional expression of α-fodrin was retained in the Aire-deficient thymus. These results suggest that Aire regulates the survival of autoreactive T cells beyond transcriptional control of self-protein expression in the thymus, at least against this ubiquitous protein. Rather, Aire may regulate the processing and/or presentation of self-proteins so that the maturing T cells can recognize the self-Ags in a form capable of efficiently triggering autoreactive T cells. With the use of inbred

Aire-deficient mouse strains, we also demonstrate the presence of some additional factor(s) that determine the target-organ specificity of the autoimmune disease caused by Aire deficiency.

Introduction

Autoimmune diseases are mediated by sustained adaptive immune responses specific for self-Ags through unknown mechanisms. Although breakdown of self-tolerance is considered to be the key event in the disease process, the mechanisms that allow the production of auto-Abs and/or autoreactive lymphocytes are largely enigmatic (1). The situation seems to have become more complicated due to the existence of multiple factors that influence the disease process, such as environmental factors, immune dysregulation and genetic predisposition. In this regard, although only a small number of genes genetically relevant to the pathogenetic processes for the development of autoimmune diseases have been found so far (2), genetic engineering of such genes in mice should enable us to establish disease models and facilitate an understanding of the disease mechanisms to a large extent. One of these genes is the autoimmune regulator (*AIRE*), mutation of which is responsible for the development of autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy (APECED: OMIM 240300) with autosomal recessive inheritance (3-6).

The AIRE gene encodes a predicted 58-kDa protein carrying a conserved nuclear localization signal, two PHD-type zinc fingers,

four LXXLL motifs or nuclear receptor interaction domains, and the recently described homogeneously staining region (HSR) and SAND domains (3, 4); the HSR and SAND domains have been suggested to function in homodimerization and DNA-binding, respectively (7, 8). Based on the fact that PHD resembles the RING finger, which can function as an E3 ubiquitin ligase, in both sequence and structure (9), we have recently found that AIRE acts as an E3 ubiquitin ligase through the N-terminal PHD domain (PHD1) (10). Because the ubiquitin-proteasome pathway plays an essential role in diverse cell functions such as cell cycle progression, signal transduction, cell differentiation, DNA repair and apoptosis (11, 12), we speculate that AIRE should play a fundamental role by facilitating polyubiquitylation of the substrate(s) in yet undetermined processes. The significance of this finding was underscored by the fact that disease-causing missense mutations in PHD1 abolished its E3 ligase activity (10).

One important aspect of AIRE in the context of autoimmunity is its limited tissue expression in medullary thymic epithelial cells (mTEC) and cells of the monocyte-dendritic cell lineage of the thymus (13, 14). Both cell types are considered to play major roles in the establishment of self-tolerance by eliminating autoreactive T cells (negative selection) (1, 15) and/or by producing

immunoregulatory T cells (Tregs), which prevent $CD4^+$ T-cell-mediated organ-specific autoimmune diseases (16, 17). For this purpose, thymic epithelial cells (TECs) have been postulated to express a set of self-Ags encompassing all of the self-Ags expressed by parenchymal organs. Supporting this hypothesis, analysis of gene expression in the thymic stroma has demonstrated that mTECs are a specialized cell type in which promiscuous expression of a broad range of peripheral tissue-specific genes is an autonomous property (18). Aire in TECs has been suggested to regulate this promiscuous gene expression (19).

Fundamental roles of Aire in the elimination of autoreactive T cells *in vivo* have been demonstrated by the use of a TCR-transgenic mouse system (20). Mice expressing hen egg lysozyme (HEL) in pancreatic β-cells driven by the rat insulin promoter (RIP) were crossed with mice expressing TCR specific for HEL, and the fate of HEL-specific T cells was monitored in either the presence or absence of Aire. Remarkably, Aire-deficient TCR-transgenic mice showed almost complete failure to delete the autoreactive (i.e., HEL-specific) T cells in the thymus (20). Because Aire-deficient mTEC showed a reduction in transcription of a group of genes encoding peripheral Ags analyzed by the gene-chip technique (19), it has been hypothesized that pathogenic autoreactive T cells could

not be eliminated efficiently due to the reduced expression of corresponding target Ags in the Aire-deficient thymus (20). However, as this transgenic study did not demonstrate the effect of Aire loss on the thymic expression of HEL, there is still a lack of experimental evidence to connect the postulated roles of Aire in the transcriptional regulation of tissue-specific Ag expression with efficient elimination of autoreactive T cells. Thus, beyond transcriptional control of self-Ags in the thymus, other mechanisms of AIRE-dependent tolerance remain to be investigated. Furthermore, the effect of Aire deficiency on the production and/or function of Tregs has not yet been fully documented (19-21). Finally, the factors contributing to the complexity of the APECED phenotype (i.e., involvement of various target organs among patients) are unknown. Although intrafamilial variation in the clinical pictures suggests that factors other than the specific *AIRE* mutations might be involved in the disease process (22), this hypothesis cannot be easily proven in human subjects. In order to approach these issues, we have generated Aire-deficient mice by gene targeting. Identification of a target Ag associated with the tissue destruction caused by Aire deficiency together with strain-dependent target-organ specificity of the autoimmune disease has suggested unique properties of AIRE in the establishment and

maintenance of self-tolerance.

Materials and Methods

Mice

Aire-deficient mice were generated by gene targeting. Briefly, the targeting vector was constructed by replacing the genomic *Aire* locus starting from exon 5 to exon 12 with the neomycin resistance gene (*neo*^{r}). The targeting vector was introduced into TT2 embryonic stem cells (H-2$^{b/k}$) (23), and the homologous recombinant clones were first identified by PCR and confirmed by Southern blot analysis. After the targeted cells had been injected into ICR 8-cell embryos (CLEA Japan, Osaka, Japan), the resulting chimeric male mice were mated with C57BL/6 females to establish the germline transmission. C57BL/6 mice, BALB/c mice and BALB/cA Jcl-nu mice were purchased from CLEA Japan. The mice were maintained under pathogen-free conditions, and handled in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine. The experiments were initiated when the mice were 8 to 12 weeks of age.

Pathology

Formalin-fixed tissue sections were subjected to H&E staining, and two pathologists independently evaluated the histology without being informed of the condition of each individual mouse.

Histological changes were scored as 0 (no change), 1 (mild lymphoid cell infiltration) or 2 (marked lymphoid cell infiltration).

Measurement of tear secretion

Measurement of tear secretion was performed as previously described (24, 25). Briefly, anesthetized mice were injected intraperitoneally with 100 μl of pilocarpine hydrochloride (1 mg/ml) to stimulate tear production. Secreted tears were absorbed every 5 min with a cotton thread treated with a pH indicator phenol red (ZONE-QUICK; Menicon, Nagoya, Japan), and the length of the red portion of the thread was measured each time. Total length of the red portion of the thread during the first 20 min after pilocarpine injection was normalized by body weight.

ELISA and Western blot analysis

Various forms of recombinant α-fodrin were expressed with pGEX-4Ts plasmids (26). Western blot analysis and ELISA for the detection of auto-Abs against various forms of recombinant α-fodrin were performed with anti-mouse IgG Ab (Vector Laboratories, Burlingame, CA), as described previously (25, 27-31). For the ELISA, absorbance values greater than the mean ± 3 S.D. in wild-type sera were considered positive. Western blot analysis of α-fodrin

expression from the proteins extracted from the thymus and lacrimal glands was performed with mouse anti-α-fodrin monoclonal Ab (Affiniti, Exeter, UK) and rabbit anti-AFN-A polyclonal Ab (25, 27-31).

Autoreactive responses against α-fodrin

For *in vitro* stimulation with α-fodrin, total splenocytes were stimulated with 10 μg/ml recombinant α-fodrin. For the last 8 h of the 32-h culture period, the cells were pulsed with [^{3}H]thymidine, and ^{3}H incorporation was determined as described previously (25).

Thymic stroma preparation

Thymic stroma was prepared as described previously with slight modification (32). Briefly, thymic lobes were isolated from three mice for each group and cut into small pieces. The fragments were gently rotated in RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated FCS (Invitrogen), 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-ME, hereafter referred to as R10, at 4 °C for 30 min, and dispersed further with pipetting to remove the majority of thymocytes. The resulting thymic fragments were digested with 0.15 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO) and 10 U/ml DNase I

(Roche Molecular Biochemicals, Mannheim, Germany) in RPMI1640 at 37 °C for 15 min. The supernatants that contained dissociated TECs were saved, while the remaining thymic fragments were further digested with collagenase IV and DNase I. This step was repeated twice, and the remaining thymic fragments were digested with collagenase IV, DNase I and 0.1 mg/ml dispase I (Roche Applied Science, Penzbeg, Germany) at 37 °C for 30 min. The supernatants from this digest were combined with the supernatants from the collagenase digests, and the mixture was centrifuged for 5 min at 450 x *g*. The cells were suspended in PBS containing 5 mM EDTA and 0.5% FCS, and kept on ice for 10 min. $CD45^-$ thymic stromal cells were then purified by depleting $CD45^+$ cells with MACS CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The resulting preparations contained approximately 60% $Ep\text{-}CAM^+$ cells and < 10% thymocytes (i.e., CD4/CD8 single-positive and CD4/CD8 double-positive cells), as determined by flow-cytometric analysis.

RT-PCR

RNA was extracted from thymic stromal cells with High Pure RNA Isolation Kit (Roche Applied Science), and made into cDNA with cDNA Cycle Kit (Invitrogen) according to the manufacturer's

instructions. The following primer pairs for the *α-fodrin* gene were used: 5'-GCTTCAAGGAGCTCTCTACC-3' and 5'-GCAGTTTGATTCCTTTCTCC-3' (encompassing *α-fodrin* exons 1-3; accession No. XM_355324), 5'-CCAGCAGCAACAATTTAATC-3' and 5'-AGCAGATTCTGGACTCCAAT-3' (encompassing the *α2-spectrin* exons 2-4; accession No. XM_207079), and 5'-GTGCAGAAATCAGCTGAGAA-3' and 5'-GCTTGTGTTTCTTCCTCAGA-3' (encompassing the *α2-spectrin* exons 24-27). PCR was carried out in a final volume of 20 μl with 1.5 U of ExTaq DNA polymerase (Takara Biomedicals, Otsu, Japan) and 250 nM each primer. Cycling conditions comprised a single denaturing step at 94 °C for 10 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min, followed by a final extension step of 72 °C for 10 min. For *β-actin*, a single denaturing step at 94 °C for 3 min was followed by 25 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 3 min (33).

Real-time PCR

Real-time PCR for quantification of *α-fodrin*, *Foxn1* and tissue-specific Ag genes was carried out with thymic stroma cDNA prepared as described above. The primers and the probes are as follows. *α2-spectrin* primers: 5'-GACAGCCAGTGATGAGTCATACAAG-3' and

5'-CACGGATTCGGTCAGCATT-3'; *α2-spectrin* probe: 5'-FAM-ACCCCACCAACATCCAGAGCAAGC-3'; *Foxn1* primers: 5'-GACATGCACCTCAGCACTCTCTA-3' and 5'-CTGATGTTGGGCATAGCTCAAG-3'; *Foxn1* probe: 5'-FAM-CCCGGCTCAAAGCCATTGGCTC-3'; *Insulin* primers: 5'-AGACCATCAGCAAGCAGGTC-3' and 5'-CTGGTGCAGCACTGATCCAC-3'; *Insulin* probe: 5'-FAM-CCCGGCAGAAGCGTGGCATT-3'; *salivary protein 1* primers: 5'-ACTCCTTGTGTTGCTTGGTGTTT-3' and 5'-TCGACTGAATCAGAGGAATCAACT-3'; *salivary protein 1* probe: 5'-FAM-TTCACCAGCAGAATCAGCAGTTCCAGAA; *C-reactive protein* primers: 5'-TACTCTGGTGCCTTCTGATCATGA-3' and 5'-GGCTTCTTTGACTCTGCTTCCA-3'; *C-reactive protein* probe: 5'-FAM-CAGCTTCTCTCGGACTTTTGGTCATGA-3'; *fatty acid binding protein* primers: 5'-CGTGTAGACAATGGAAAGGAGCT-3' and 5'-AAGAATCGCTTGGCCTCAACT-3'; *fatty acid binding protein* probe: 5'-FAM-TCATTACCAGAAACCTCTCGGACAGCA-3'; *glutamic acid decarboxylase 67* primers: 5'-TCCTCCAAGAACCTGCTTTCC-3' and 5'-GCTCCTCCCCGTTCTTAGCT-3'; *glutamic acid decarboxylase 67* probe: 5'-FAM-CCGACTTCTCCAACCTGTTTGCTCAAGA-3'. *Foxp3* expression was examined with cDNAs prepared from splenocytes ($CD4^+CD25^+$ or $CD4^+CD25^-$) and total thymus. The primers, the probes and the reactions used for *Foxp3* and *Hprt* were those

described previously (33, 34).

Thymus grafting

Thymus grafting was performed as previously performed (33). Briefly, thymic lobes were isolated from embryos at 14.5 days post-coitus, and then cultured for 4 days on Nuclepore filters (Whatman, Clifton, NJ) placed on R10 containing 1.35 mM 2'-deoxyguanosine (Sigma-Aldrich). Five pieces of thymic lobes were grafted under the renal capsule of BALB/c *nude* mice. After 6 to 8 weeks, reconstitution of peripheral T cells was determined by flow-cytometric analysis with anti-CD4 (clone GK1.5; BD PharMingen, San Diego, CA) and anti-CD8 (clone 53-6.7; BD PharMingen) mAbs, and then the thymic chimeras were used for analysis.

Immunohistochemistry

Immunohistochemical analysis of the thymus was performed as described previously (35, 36). For the detection of auto-Abs, mouse serum was incubated with various organs obtained from Rag2-deficient mice. FITC-conjugated anti-mouse IgG Ab (Southern Biotechnology Associates, Birmingham, AL) was used for the detection (33).