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The possible etiopathogenic genes of Sjögren's syndrome

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Abstract

Sjögren's syndrome is a chronic autoimmune disease characterized by focal lymphocytic infiltration of lacrimal and salivary glands, but the precise mechanism of this syndrome is unclear. To clarify the pathogenesis of Sjögren's syndrome, the related genes must be identified. In the present study, we investigate the increased expression of genes and molecules related to Sjögren's syndrome and present our findings of cDNA microarray analysis in the mouse model. Furthermore, we present the results of immunohistochemical analysis of salivary glands in the mouse model and patients with Sjögren's syndrome. This approach might open a new discussion of the existence of principal pathogenic molecules in Sjögren's syndrome.

Keywords: Sjögren's syndrome; MRL/lpr mice; NFS/sld mice; cDNA microarray; Human homologue of SS related genes

Contents

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1. Mouse model of Sjögren's syndrome

Sjögren's syndrome (SS) is an autoimmune disease characterized by the massive infiltration of lymphocytes into exocrine glands, such as salivary and lacrimal glands, and the subsequent destruction of these exocrine glands. Like other autoimmune diseases, the etiology of SS remains unclear, but previous studies suggest the involvement of hereditary and environmental factors in the onset and progression of the disease. The disease is usually benign and many patients live a typical lifespan. However, the most common symptoms, dry eyes and dry mouth, are problematic and deeply influence patients' quality of life. In addition to these relatively benign manifestations, abnormalities of more vital organs such as renal tubular acidosis, interstitial pulmonary fibrosis, and central nervous system involvement have been demonstrated [1–4]. Therefore, it is important to determine the etiology of SS for the improved management of the disease.

An animal model is one of the most useful tools for studying the pathogenesis of SS; several mouse models have been generated and extensively studied. Among these models, the MRL/lpr mouse bearing the *lpr* gene with a deletion of Fas antigen spontaneously develops systemic vasculitis, glomerulonephritis, arthritis, and sialoadenitis. High levels of autoantibodies, immune complexes, and rheumatoid factor have also been observed in this mouse model [5,6]. Inflammation of the salivary glands in the MRL/lpr mouse is widely accepted as a pathogenic model for human secondary SS [7]. Although the fundamental molecular abnormality in the MRL/lpr mouse model directly depends on the *lpr* gene, the extent of the phenotype and the timing of onset are strongly influenced by background genes [8–10].

The NFS/sld mutant mouse is an animal model of primary SS that bears an autosomal recessive gene that arrests sublingual gland differentiation. Autoimmune sialoadenitis develops when NFS/sld mice undergo a thymectomy 3 days after birth without any immunization (Tx-NFS/sld mice). While no significant inflammatory lesions are observed in other organs or in NFS/sld mice that do not undergo a thymectomy (non-Tx-NFS/sld mice), significant inflammatory changes occur in the salivary glands of Tx-NFS/sld mice 4 weeks after their thymectomy [11,12].

2. cDNA Microarray analysis

Gene expression analysis provides an important perspective on unknown biological phenomena. The following methods are established and applied for basic and clinical studies: differential display [13], suppression subtractive hybridization [14], cDNA microarray hybridization [15], and serial analysis of gene expression (SAGE) [16]. A microarray system is a powerful tool for analyzing the expression profile of thousands of genes in a wide range of biological systems. Recently, microarray analysis has been applied for the research of various clinical disorders such as lymphoma, Huntington's disease, and myocardial infarction, and disease-related genes were isolated in some of these disorders [17–21].

In the present study, we isolated genes that contribute to the progression of SS, using mRNA from SS model mouse salivary glands and an in-house cDNA microarray, and identified up-regulated genes.

3. Sjögren's syndrome-related genes and molecules

To investigate the gene expression profile in SS, we examined the mRNAs of the MRL/lpr and NFS/sld mouse salivary glands using cDNA microarrays. We arrayed a set of 4608 cDNA clones derived from oligo-capped mouse brain, fetus, kidney, and spleen. The most aggressive inflammation in the salivary gland of MRL/lpr mouse occurs at the age of 12–16 weeks [8,22], so we compared the mRNAs of MRL/lpr and MRL/++ mouse salivary glands at the age of 16 weeks. We identified 15 highly expressed genes, *IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mpt1*, *Laptm5*, *Gnai2*, *vimentin*, *UCP2*, *saposin*, *Trt*, *laminin receptor 1*, *and HSP 70 cognate*, in the salivary gland of MRL/lpr mouse by cDNA microarray analysis, which were likely to be SS-related genes [23] (Table 1).

We performed reverse transcription-polymerase chain reaction amplification to confirm the high expression of the following 15 genes. High expression was verified in 11 of the 15 genes: *IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *vimentin*, *Mel-14 antigen*, *cathepsin B*, *mpt1*, *Laptm5*, *Gnai2*, and *UCP2*. Five of these genes (*caspase 3*, *Ly-6C*, *vimentin*, *Mel-14 antigen*, and *cathepsin B*) have already been recognized in patients with SS or the SS mouse model [24–29].

Table 1
Highly expressed genes in MRL/lpr mice salivary gland in comparison with MRL/++

Accession No.	Name of genes	Fold change[a]
NM_009810	Mus musculus *caspase 3*	2.31
M18466	Mouse lymphocyte differentiation antigen *Ly-6C.2*	2.75
M26251	Mouse *vimentin*	2.21
M25324	Mouse peripheral lymph node-specific homing receptor (*MEL-14* antigen)	3.50
NM_007798	Mus musculus *cathepsin B*	1.84
AF006467	Mus musculus membrane-associated phosphatidylinositol transfer protein (*mpti*)	1.98
NM_010686	Mus musculus lysosomal-associated protein transmembrane 5 (*Laptm5*)	2.16
NM_008138	Mus musculus guanine nucleotide binding protein, alpha inhibiting 2 (*Gnai2*)	1.93
U69135	Mus musculus *UCP2*	2.06
S36200	Mouse saposin=sphingolipid activator protein	1.88
NM_009429	Mus musculus translationally regurated transcript (*Trt*)	1.82
NM_011029	Mus musculus laminin receptor 1 (*Lamr1*)	2.02
M19141	Mouse heat shock protein 70 cognate	1.61
AF175292	Mus musculus neuronal *IL-16*	2.20
NM_027817	GRB2-related adaptor protein (*Grap*), mRNA	1.85

[a] The averages of the fold change based on the normalized microarray fluorescent data of MRL/lpr compared to MRL/++ ($n=8$).

Although a high expression of *caspase 3* has been reported in the NOD mouse model of SS [24], the MRL/lpr mouse is Fas-deficient and thus lacks Fas/Fas ligand pathway-dependent apoptosis. This suggests that Fas/Fas ligand pathway-independent apoptosis, such as perforin-or granzyme-dependent apoptosis [30], is induced in MRL/lpr mouse salivary glands. One of the adaptor molecules, Grap, effectively delivers signals from the immune cell surface to a downstream functional molecule. Grap has a structural arrangement of an SH3-SH2-SH3 domain, which is similar to that of other immune cell adaptor molecules such as Grb2, Gads, and Grap2 [31]. Grap is known to be specifically expressed in lymphoid tissues, and structurally resembles Grb2 more than other Grb2 family molecules in that Grap does not have the proline-rich motif. By immune cell activation, Grap binds to phosphorylated tyrosine of the local area transport (LAT) at its SH2 region, and further binds to Son of sevenless (Sos) in a manner similar to that of Grb2. Further down-stream events remain unknown. We have observed that the expression of Grap in the salivary glands of the model mice was higher than that of the control mice. Furthermore, we have identified 7 genes in the spleen of MRL/lpr mice not found in the spleen of MRL/+ mice using the mouse spleen cDNA microarray chip [32] (Table 2). Namely, the *Grap* gene was commonly up-regulated in the spleen and salivary glands from MRL/lpr mice [32]. Immunohistochemical staining in the salivary gland revealed substantial differences between MRL/lpr and MRL/++ in the expression of mouse Grap. Furthermore, the immunohistochemical staining of specimens from 3 patients with SS and 2 controls (subjects with salivary cysts) indicated that the human homologue of Grap was expressed on ductal cells and on certain infiltrating cells in patients with SS, but very weakly in the controls [32]. These results may suggest that in diseased salivary glands and spleen, enhanced stimulation of T cell receptor augments signal transduction to downstream molecules associated with apoptosis. Further detailed analysis of the Grb2 family may clarify the regulation of T cell differentiation and apoptosis in SS.

Table 2
Highly expressed genes in MRL/lpr mice spleen in comparison with MRL/++

Accession No.	Name of genes	Fold change[a]
U88682	Mouse anti-DNA antibody heavy chain variable region mRNA	2.88
XM_134565	Mouse similar to Gag-Pol polyprotein mRNA	1.93
M16072	Mouse Ig active gamma-2a H-chain V-Dsp2.2-J2-C mRNA	1.64
BC036286	Mouse myeloid/lymphoid or mixed-lineage leukemia 5, mRNA	2.17
NM_025408	Mouse phytoceramidase, alkaline (Phca)mRNA	3.23
X76772	Mouse mRNA for ribosomal protein S3	1.56
NM_027817	Mus musculus GRB2-related adaptor protein (*Grap*), mRNA	2.82

[a] The averages of the fold change based on the normalized microarray fluorescent data of MRL/lpr compared with MRL/+ ($n=6$).

To our knowledge, the remaining five genes (*mptl, Laptm5, UCP2 Gnai2* and *IL16*) have not been identified previously as SS-related genes. *Mptl* was cloned as a mouse homologue of Drosophila retinal degeneration B (rdgB), and the mptl protein has been predicted to be a membrane-bound phosphatidylinositol transfer protein (PITP) [33], which transports phosphatidylinositol (PI) through the aqueous phase from one membrane compartment to another and functions as a cofactor for the synthesis of phosphatidylinositol bisphosphate (PIP2) [34]. Given that the constitutive turnover of PI is markedly augmented in some subsets of T lymphocytes in MRL/lpr mice [35], it is fair to speculate that such T cells accumulate and are related to the pathogenesis of SS. *Laptm5* is highly expressed in adult hematopoietic organs such as bone marrow, spleen, thymus, lymph nodes, and peripheral blood leukocytes [36]. The high expression of *Laptm5* in our MRL/lpr mouse model of SS could be due to an increased number of lymphocytes infiltrating the salivary glands. Further experimental studies are required to clarify the role of *Laptm5* in the pathogenesis of SS. The mitochondrial protein known as uncoupling protein 2 (*UCP2*) is highly expressed in the spleen and macrophages. A recent report suggests that *UCP2* plays a role in limiting macrophage-mediated immunity [37]. The expression of *UCP2* is induced by TNF-α [38] and the expression of TNF-α is increased in MRL/lpr mice [39]. These combined findings suggest that increased TNF-α in MRL/lpr mouse salivary glands could contribute to the up-regulation of *UCP2* and subsequent disease progression.

We then examined the expression of these genes in the salivary glands of MRL/lpr mice and NFS/sld mice that had undergone a thymectomy (Tx-NFS/sld), a new model for primary SS, by using real-time-quantitative reverse transcription-polymerase chain reaction analysis. The expression of 11 genes (*IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mptl*, *Laptm5*, *Gnai2, vimentin,* and *UCP2)* was higher in MRL/lpr mice than in MRL/++ mice and the expression of 9 genes (*IL-16*, *Grap*, *caspase3*, *Ly6c2, Mel-14 antigen*, *cathepsinB*, *mptl Laptm5,* and *Gnai2)* was higher in the Tx-NFS/sld mice than in the control mice that did not undergo thymectomy. In addition, the fetus microarray analysis demonstrated that the *Laptm5* gene was also highly expressed in the salivary glands of Tx-NFS/sld mice. Furthermore, immunohistochemical studies showed that mouse and human *Laptm5* protein antigens were expressed on certain infiltrated lymphocytes and on ductal cells in the salivary glands from the SS mouse model and patients with SS, but very weakly in control subjects. These results suggest that some apoptosis-related genes might be responsible for the pathogenesis of organ-specific autoimmune lesions in SS (Arthritis Rheumatism Vol. 9, S251, 2001, Arthritis and Rheumatism Vol. 50 S577 2004).

These early findings confirm the excellent specificity and reproducibility of our cDNA microarray analysis for identification of disease-related genes. A microarray system can handle thousands of genes and help in the extraction of genes that have significant relationships to the stages of a disease. In addition, an in-house cDNA microarray is advantageous in allowing the exchange of arrayed genes and assaying specific disease-related genes on one glass slide, which is useful for the diagnosis and prediction of clinical stages.

In the present study, we isolated nine SS-related genes on a cDNA microarray using the MRL/lpr SS mouse model. Further studies may allow the identification of other SS-related genes, thus allowing the performance of clustering analysis, which could provide useful information about classification of the disease, clinical course, stage of the disease, and selection of a suitable treatment. The combination of an in-house microarray and the use of an animal model is a suitable strategy for exploring a gene expression profile and should gradually evolve into a system useful for clinical investigation.

Take-home message

- The combination of cDNA microarray and use of animal models is a suitable strategy for exploring a gene expression profile and should gradually evolve into a system useful for clinical investigation.
- Fifteen highly expressed genes, *IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mptl*, *Laptm5*, *Gnai2, vimentin*, *UCP2*, *saposin*, *Trt*, *laminin receptor 1*, and *HSP 70 cognate*, in the salivary gland of MRL/lpr mouse by cDNA microarray analysis were identified.
- The expression of 9 genes, *IL-16*, *Grap*, *caspase3*, *Ly6c2, Mel-14 antigen*, *cathepsin B*, *mptl Laptm5*, and *Gnai2,* was higher in the salivary gland of

thymectomized-NFS/sld mice than in the control mice that did not undergo thymectomy.

- Five genes, *mptl, Laptm5, UCP2 Gnai2* and *IL16*, have not been identified previously as SS-related genes.
- Human *Grap* and *Laptm5* protein antigens were expressed on certain infiltrated lymphocytes and on ductal cells in the salivary glands from patients with SS, but very weakly in control subjects.

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Endometriosis and systemic lupus erythematosus: a comparative evaluation of clinical manifestations and serological autoimmune phenomena.

Due to evidences suggesting association between endometriosis (EM) and systemic lupus erythematosus (SLE), Pasoto SG. et al. (Am J Reprod Immunol 2005;53:85–93), have performed a comparative evaluation of clinical and humoral immunologic abnormalities in both diseases. Forty-five women with histologically confirmed pelvic EM, 21 healthy-women and 15 female SLE-patients without surgically confirmed EM were prospectively evaluated. None of the EM-patients fulfilled criteria for SLE. However, EM-patients presented higher frequencies of arthralgia (62%) and generalized myalgia (18%) compared to that of normal-controls (24%, $p = 0.04$) but comparable with SLE-patients. Antinuclear antibodies (ANA) were detected in 18% of EM-patients, as compared with healthy-women ($p = 0.01$). Anti-Ro and anticardiolipin antibodies were more often in SLE (40%, 33%) than in EM-patients (2%, $p < 0.001$ and 9% $p = 0.04$). Elevated immune-complexes and low total complement were also more frequent in SLE patients. The data indicate differences of ANA antigenic specificity and complement consumption between EM and SLE. The high prevalence of generalized musculoskeletal complaints in EM justifies a multidisciplinary approach.

Two cases of antinuclear antibody negative lupus showing increased proportion of B cells lacking RP105.

B cells lacking RP105 molecule, a member of the Toll-like receptor family, were increased in the peripheral blood of 2 patients with antinuclear antibody (ANA) negative systemic lupus erythematosus (SLE). The increased proportion of RP105-lacking B cells was associated with disease activity in patients with ANA-negative SLE. Koarada S. et al (J Rheumatol 2005; 32:562-4) suggested that when there are no significant serological markers for SLE, analyses of expression of RP105 may be helpful in evaluation of activity in ANA-negative SLE. Thus they describe a new approach, using phenotyping of B cells, to evaluate activity of ANA-negative SLE.

BRIEF COMMUNICATIONS

nature genetics

Mutations in the gene encoding fibroblast growth factor 10 are associated with aplasia of lacrimal and salivary glands

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Autosomal dominant aplasia of lacrimal and salivary glands (ALSG; OMIM 180920 and OMIM 103420) is a rare condition characterized by irritable eyes and dryness of the mouth. We mapped ALSG to 5p13.2–5q13.1, which coincides with the gene fibroblast growth factor 10 (*FGF10*). In two extended pedigrees, we identified heterozygous mutations in *FGF10* in all individuals with ALSG. *Fgf10*$^{+/-}$ mice have a phenotype similar to ALSG, providing a model for this disorder. We suggest that haploinsufficiency for FGF10 during a crucial stage of development results in ALSG.

ALSG has variable expressivity, and affected individuals may have aplasia or hypoplasia of the lacrimal, parotid, submandibular and sublingual glands and absence of the lacrimal puncta[1]. The disorder is characterized by irritable eyes, recurrent eye infections, epiphora (constant tearing) and xerostomia (dryness of the mouth), which increases the risk of dental erosion, dental caries, periodontal disease and oral infections[2]. Individuals affected with ALSG are sometimes misdiagnosed with the more prevalent disorder Sjögren syndrome, an autoimmune disorder characterized by keratoconjunctivitis sicca and xerostomia[3]. Both sporadic and familial cases of ALSG have been described[2,4,5]. We recently identified two extended families of Swedish origin with ALSG (**Fig. 1a**). The phenotypes of the affected individuals are summarized in **Supplementary Table 1** online. In total, 16 individuals from both families were diagnosed with ALSG (**Supplementary Methods** online). We investigated the lacrimal and major salivary glands by magnetic resonance imaging (**Supplementary Fig. 1** online), which showed aplasia or hypoplasia of several major salivary glands in all affected individuals and absent or hypoplastic lacrimal glands in 13 of 14 affected individuals. We observed absence of one or several lacrimal puncta in 13 of 14 affected individuals. We observed no other abnormalities, and the affected individuals had normal lifespans.

Inheritance of ALSG in both families is autosomal dominant, and the segregation pattern suggested full penetrance. A genome-wide screen with 400 polymorphic microsatellite markers showed linkage of ALSG to 5p13.2–5q13.1 flanked by microsatellite markers *D5S395* and *D5S2046* (**Fig. 1a**). We obtained a maximum cumulative lod score of 5.72 ($\theta = 0$) at the marker locus *D5S398* for both families (**Supplementary Table 2** online). The gene fibroblast growth factor 10 (*FGF10*) is located in the linked region[6]. Mouse FGF10, which is 93% identical to human FGF10, is crucial for the development of several organs, including lacrimal and salivary glands[7–9]. *Fgf10*$^{-/-}$ mice die shortly after birth[9,10]. No abnormalities have been described in *Fgf10*$^{+/-}$ mice.

We considered *FGF10* as a candidate gene for ALSG. Sequence analysis of the three exons of *FGF10* in samples from family 1 showed no alterations compared with sequences in the National Center for Biotechnology Information database. To identify deletions, we genotyped the family members for SNPs and microsatellite markers in *FGF10*. The affected members of family 1 were hemizygous with respect to two dinucleotide repeats (TA53 and CA17) and three SNPs (rs10060548, rs6881797 and rs2290070; **Fig. 1b,c**). After genotyping, we characterized the deletion breakpoint by long-range PCR and sequencing across the breakpoint (**Supplementary Fig. 2** online). We determined the size of the deletion to be 53 kb, including exons 2 and 3, without the involvement of any flanking genes (**Fig. 1b**). In family 2, DNA sequence analysis of *FGF10* identified a heterozygous stop mutation in exon 3 (R193X; 577C→T) resulting in a predicted truncated protein in the four affected members (**Fig. 1d**).

We then reexamined *Fgf10*$^{+/-}$ mice, which were previously described as apparently normal[8,10]. We dissected adult mice and carried out a macroscopical and histological examination of the lacrimal and salivary gland apparatuses. *Fgf10*$^{+/-}$ mice had aplasia of lacrimal glands and hypoplasia of salivary glands (**Fig. 2**). These findings are consistent with the phenotype of individuals with ALSG. Other internal organs, including lung, liver, spleen, heart, stomach, thyroid, pancreas, intestines and ovaries, were macroscopically normal in *Fgf10*$^{+/-}$ mice.

To clarify whether *FGF10* mutations cause dry eyes and dry mouth in sporadic cases with symptoms identical to those of individuals with ALSG, we screened DNA samples from 74 individuals for mutations in *FGF10*. These individuals had been evaluated and diagnosed with dry eyes and/or dry mouth, without fulfilling the criteria for Sjögren syndrome[11,12]. We found no sequence alterations in the coding region of *FGF10* in samples from these individuals,

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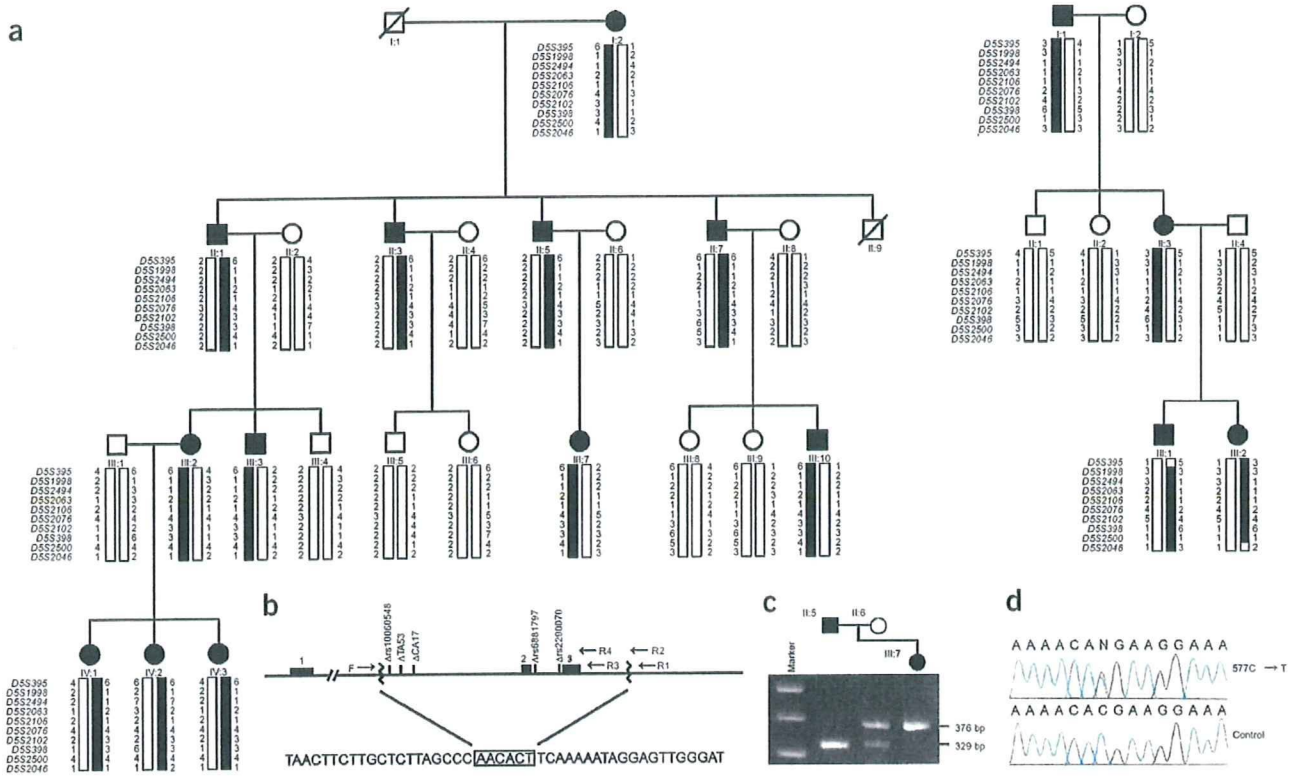

Figure 1 ALSG mapping. **(a)** Two pedigrees segregating for ALSG. Marker haplotypes on chromosome 5p13.2–5q13.1 that are linked to ALSG are indicated by black bars. **(b)** Schematic overview of *FGF10* and the 53-kb deletion inherited with ALSG in family 1 (figure not drawn to scale). Black boxes denote exons 1–3, and wavy vertical lines indicate deletion breakpoints. Genomic sequence spanning the breakpoint is shown. **(c)** Genotyping of the SNP rs6881797, located in the deletion found in family 1 and 37 bp 3′ of exon 2, by digestion with *Bsr*I. Undigested PCR product (376 bp) corresponds to the T allele and digested PCR product (329 bp) corresponds to the A allele. The absence of a paternal A allele in individual III:7 in family 1 indicates hemizygosity with respect to rs6881797. **(d)** The upper sequence chromatogram illustrates the heterozygous (R193X; 577C→T) mutation found in the affected members of family 2. The lower sequence chromatogram illustrates the corresponding normal sequence.

suggesting that mutations in *FGF10* are uncommon in individuals with unspecific sicca syndromes.

In family 1, the affected individuals are hemizygous with respect to exons 2 and 3 of *FGF10*. In family 2, the premature stop codon (R193X; 577C→T) in exon 3 predicts a truncated protein with a loss of 16 amino acids. The truncation abolishes one predicted cAMP- and cGMP-dependent protein kinase phosphorylation site (residues 194–197) and one predicted *N*-linked glycosylation site (residues 196–198)[13]. Furthermore, the truncation eliminates one of the sites for the interaction between FGF10 and fibroblast growth factor receptor 2b (FGFR2b) at residues 202 and 204 (ref. 14). If produced, the truncated FGF10 is probably unstable or nonfunctional. Both mutations in *FGF10* that we identified are consistent with the idea that haploinsufficiency with respect to FGF10 underlies ALSG.

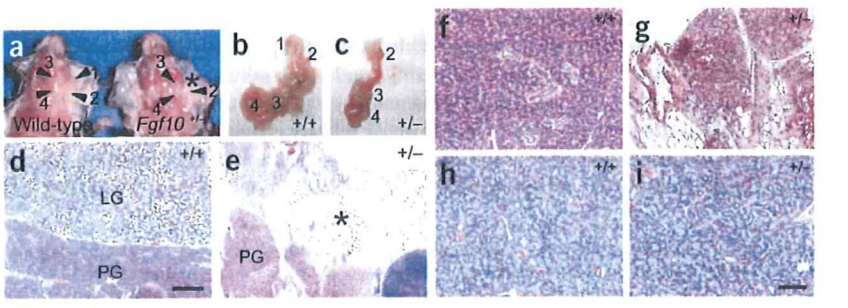

Figure 2 Salivary and lacrimal gland apparatuses of wild-type and *Fgf10*$^{+/-}$ mice. **(a)** Macroscopic examination of wild-type (left) and *Fgf10*$^{+/-}$ (right) adult mice. Ventral view of the mandibular region. 1, lacrimal; 2, parotid; 3, sublingual; 4, submandibular glands. The asterisk indicates the expected site for the lacrimal gland, which was absent in the heterozygote. Other glands were hypoplastic in the heterozygote. **(b,c)** Dissected salivary and lacrimal glands from the wild-type **(b)** and *Fgf10*$^{+/-}$ **(c)** mice. The salivary glands from the *Fgf10*$^{+/-}$ mouse were hypoplastic and the lacrimal gland was absent. **(d–i)** Histology of wild-type **(d,f,h)** and *Fgf10*$^{+/-}$ **(e,g,i)** glands. **(d)** Wild-type lacrimal (LG) and parotid (PG) glands. **(e)** The lacrimal gland was replaced by adipose tissue (asterisk) in the *Fgf10*$^{+/-}$ mouse. **(f)** Wild-type parotid gland. **(g)** Parotid gland from *Fgf10*$^{+/-}$ mouse, which appears atrophic. **(h,i)** Submandibular glands have similar histology in the wild type and heterozygote, as did sublingual glands (not shown). Scale bars: **d** (for panels **d** and **e**), 0.5 mm; **i** (for panels **f–i**), 0.1 mm.

The clinical examinations and medical histories of the affected family members illustrate that one intact copy of *FGF10* is sufficient for development of essential organs in humans. The restricted phenotype associated with heterozygosity with respect to *FGF10* in both humans and mice suggests that the response to FGF10 is dosage-sensitive. This is probably related to a specific embryonic stage and occurs at the site of lacrimal and salivary gland formation. A possible explanation for the absence of generalized effects in *FGF10* hemizygotes is a functional overlap with other FGFR2b ligands, such as FGF1, FGF3 and FGF7 (ref. 15).

The identification of mutations in *FGF10* as causing ALSG will hopefully result in increased diagnostic accuracy. In a larger context, this report clarifies the phenotypic effects of mutations in *FGF10* and may lead to a better understanding of the mechanisms involved in lacrimal and salivary gland formation.

We obtained informed consent from all participants in the study under a protocol approved by the Ethical Committee at Uppsala University or by the collaborating Universities.

URLs. Primer 3 is available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/. The National Center for Biotechnology Information Entrez Genome Map Viewer is available at http://www.ncbi.nlm.nih.gov/mapview. The Ensembl Human Genome Server database is available at http://www.ensembl.org/. The Genome Database is available at http://www.gdb.org/.

Accession numbers. GenBank: human *FGF10*, NM_004465; human chromosome 5 clones containing *FGF10*, AC093537.2 and AC093289.2. GenBank Protein: human FGF10, NP_004456.1.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Development of Autoimmunity against Transcriptionally Unrepressed Target Antigen in the Thymus of Aire-Deficient Mice[1]

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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. *The Journal of Immunology,* **2005, 174: 1862–1870.**

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*)[3] mutation, which is responsible for the development of autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy (APECED; Online Mendelian Inheritance in Man 240300) with autosomal recessive inheritance (3–6).

The *AIRE* gene encodes a predicted 58-kDa protein carrying a conserved nuclear localization signal, two plant homeodomain (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 polyubiquitinylation of the substrate(s) in yet undetermined processes. The significance

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[3] Abbreviations used in this paper: AIRE, autoimmune regulator; APECED, autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy; TEC, thymic epithelial cell; mTEC, medullary TEC; PHD, plant homeodomain; HEL, hen egg lysozyme; 3d-Tx mice, mice thymectomized 3 days after birth; SS, Sjögren's syndrome; Treg, immunoregulatory T cell; BM, bone marrow.

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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 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. 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), 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 8–12 wk 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 i.p. 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), 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 autoAbs against various forms of recombinant α-fodrin were performed with anti-mouse IgG Ab (Vector Laboratories), as described previously (25, 27–31). For the ELISA, absorbance values greater than the mean $\pm$ 3 SD 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 mAb (Affiniti) 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) 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) and 10 U/ml DNase I (Roche Molecular Biochemicals) in RPMI 1640 at 37°C for 15 min. The supernatants that contained dissociated TECs were saved, whereas 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) 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 $\times$ *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) according to the manufacturer's instructions. The resulting preparations contained ~60% Ep-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′-GCTTCAAGGAG CTCTCTACC-3′ and 5′-GCAGTTTGATTCCTTTCTCC-3′ (encompassing *α-fodrin* exons 1–3; accession no. XM_355324), 5′-CCAGCAGCAA CAATTTAATC-3′ and 5′-AGCAGATTCTGGACTCCAAT-3′ (encompassing the *α2-spectrin* exons 2–4; accession no. XM_207079), and 5′-GTG CAGAAATCAGCTGAGAA-3′ and 5′-GCTTGTGTTTCTTCCTCAGA-3′ (encompassing the *α2-spectrin* exons 24–27). PCR was conducted in a final volume of 20 μl with 1.5 U of ExTaq DNA polymerase (Takara Biomedicals)

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 conducted 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 postcoitus, and then cultured for 4 days on Nucleopore filters (Whatman) 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 6–8 wk, reconstitution of peripheral T cells was determined by flow cytometric analysis with anti-CD4 (clone GK1.5; BD Pharmingen) 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) was used for the detection (33).

Isolation and functional analysis of Tregs

Spleen cell suspensions were stained with FITC-conjugated anti-CD25 (clone 7D4) and PE-conjugated anti-CD4 (clone H129.19) (BD Pharmingen), and sorted by FACS (ALTRA; Beckman Coulter) as described previously (37). The purity of the $CD25^-$ and $CD25^+CD4^+$ populations was >90 and 95%, respectively. Spleen cells sorted as described above were cocultured with RBC-lysed and irradiated (15 Gy) spleen cells (5×10^4) from wild-type mice as APC for 3 days in 96-well round-bottom plates in R10. Anti-CD3 mAb (clone 145-2C11) (Cedarlane Laboratories) at a final concentration of 10 μg/ml was added to the culture for stimulation, and ^3H incorporation during the last 6 h of culture was measured.

Results

Development of Sjögren's syndrome (SS)-like pathologic changes in exocrine organs from Aire-deficient mice

To investigate the roles of AIRE in the establishment and maintenance of self-tolerance in vivo, we generated Aire-null mutant mice. To this end, we deleted a large proportion of the known functional domains of *Aire* including SAND, PHD1, and PHD2 (6) (Fig. 1*A*). The correct targeted event was confirmed by Southern blot analysis and genomic PCR of material from the gene-targeted

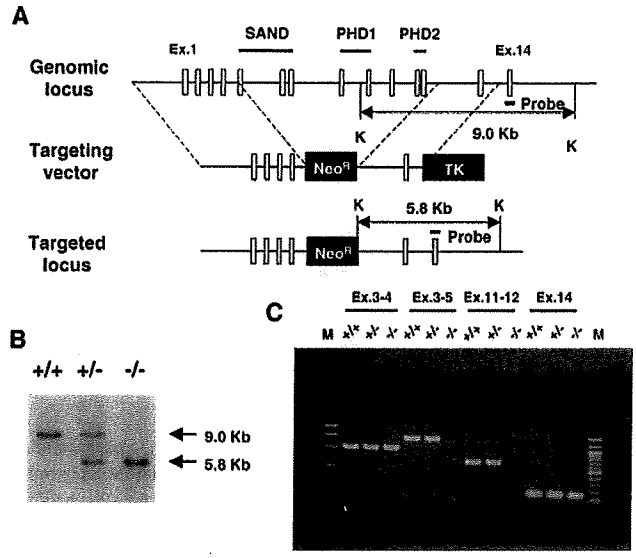


FIGURE 1. Generation of Aire-deficient mice. *A*, Targeted disruption of the gene encoding *Aire* by homologous recombination. K, *Kpn*I restriction site. *B*, Southern blot analysis of genomic DNA from offspring of heterozygous Aire-deficient mouse intercrosses. Tail DNA was digested with *Kpn*I and hybridized with a probe shown in *A*. *C*, Detection of genomic fragments of the *Aire* locus by PCR. Sequences spanning exons 5 and 12 were not amplified in tail DNA of homozygous Aire-deficient mice.

mice (Fig. 1, *B* and *C*). Offspring homozygous for Aire deficiency were born in the numbers expected from the heterozygous crossing, and homozygous Aire-deficient mice were grossly normal. Although both male and female homozygous Aire-deficient mice are fertile when crossed with wild-type mice, homozygous crossing produced offspring only occasionally (F. Kajiura and M. Matsumoto, unpublished observation). Total spleen cell numbers and total thymocyte numbers were indistinguishable between control and Aire-deficient mice. Flow cytometric analysis showed similar expression of B220, CD3, CD4, and CD8 in the spleen and thymus of control and Aire-deficient mice. Proliferative responses and Ig production from the B cells after various stimuli, and proliferative responses and IL-2 production from the T cells stimulated with anti-CD3 mAb, were also unchanged by the Aire deficiency (S. Sun and M. Matsumoto, unpublished observation).

To assess the impact of Aire deficiency on the breakdown of self-tolerance, we inspected various organs (i.e., salivary glands, lacrimal glands, thyroid, heart, lung, liver, stomach, pancreas, kidney, small intestine, testis, and ovary) from Aire-deficient mice of original mixed background (i.e., $H\text{-}2^{b/k} \times H\text{-}2^{b}$). The most marked changes were evident in the lacrimal glands (Fig. 2, *A* and *B*); all the Aire-deficient mice showed infiltration of many lymphoid cells in the lacrimal glands, whereas no such changes were observed in the control mice. We also observed infiltration of many lymphoid cells in the parotid glands (8 of 8 Aire-deficient mice) and submandibular glands (10 of 16 Aire-deficient mice) (Fig. 2*A*). Consistent with these SS-like pathologic changes in exocrine organs from Aire-deficient mice, secretion of tears per unit of mouse body weight was decreased in the affected mice (0.89 ± 0.33 mm/20 min/body weight (g) from control mice ($n = 5$) vs 0.46 ± 0.08 mm/20 min/body weight (g) from Aire-deficient mice ($n = 4$); $p < 0.05$). In 1 of 10 Aire-deficient mice, lymphoid cell infiltration in either the stomach or pancreas was also observed. There were no obvious pathologic changes in other organs from Aire-deficient mice during follow-up to the age of 8 mo.

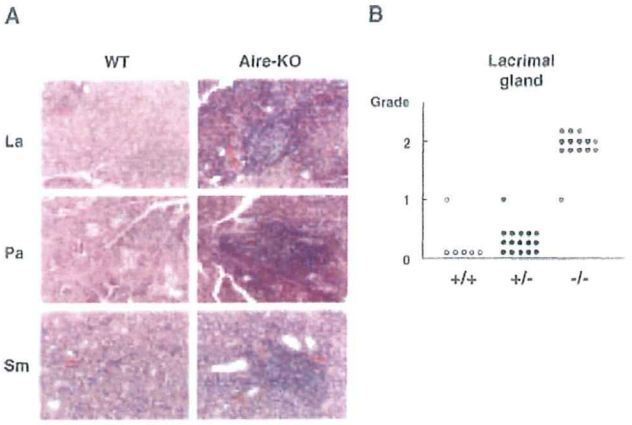


FIGURE 2. Development of organ-specific pathologic changes in Aire-deficient mice. *A*, Aire-deficient mice exhibited many infiltrating lymphoid cells in the lacrimal gland (La), parotid gland (Pa), and submandibular gland (Sm). In contrast, these changes were scarcely observed in control mice. Original magnification, ×100. *B*, Histological changes in H&E-stained tissue sections were scored as 0 (no change), 1 (mild lymphoid cell infiltration), or 2 (marked lymphoid cell infiltration). One mark corresponds to one mouse analyzed.

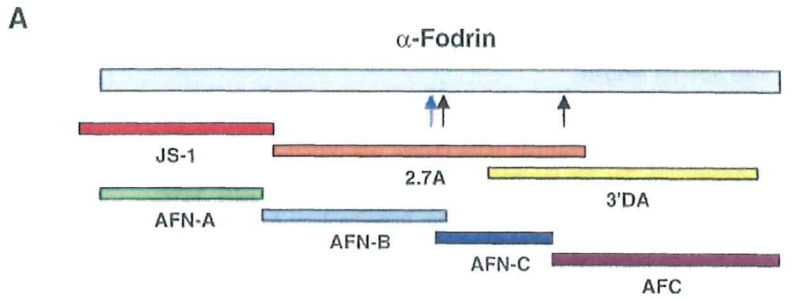
Autoreactive responses against α-fodrin in Aire-deficient mice

We have previously reported that NFS/*sld* mutant mice thymectomized 3 days after birth (3d-Tx) exhibit SS-like phenotypes with autoreactivity against α-fodrin, a ubiquitously expressed actin-binding protein (27, 38). Because of the similarity of SS-like phenotypes between Aire-deficient mice and the 3d-Tx-SS model, we investigated whether Aire-deficient mice exhibit autoreactivity against α-fodrin. We first tested the production of auto-Ab against various forms of recombinant α-fodrin in sera from Aire-deficient mice using Western blot analysis (Fig. 3, *A* and *B*). Sera from 3d-Tx mice showed reactivity predominantly against the JS-1 fragment (27). Four of five Aire-deficient mice showed reactivity against 2.7A, and two mice showed reactivity against 3′DA (Fig. 3*B*). Sera from control mice showed no such reactivities. Production of auto-Ab against α-fodrin in Aire-deficient mice was also evaluated by ELISA using additional forms of recombinant α-fodrin (31) and larger numbers of mice. Ten of 11 Aire-deficient mice showed significantly higher reactivities against at least one form of recombinant α-fodrin fragment compared with those from 11 control mice (Fig. 3*C*). Interestingly, each Aire-deficient mouse showed reactivity against different forms of α-fodrin.

We also confirmed the development of autoimmunity against α-fodrin using splenocytes from Aire-deficient mice (25). Such splenocytes cultured with recombinant α-fodrin showed significant proliferative responses; four Aire-deficient mice tested showed a response to 2.7A, but not to JS-1, whereas no such reactivities were observed from age-matched control mice (Fig. 4).

Unrepressed expression of corresponding target Ag in Aire-deficient thymus

The mechanism controlling the thymic microenvironment necessary for the establishment of self-tolerance in an Aire-dependent manner is of considerable interest. It has been suggested that "promiscuous" expression of a broad range of peripheral tissue-specific genes by TECs is essential for establishing self-tolerance (18), and Aire has been implicated in the control of this promiscuous gene expression through a transcriptional mechanism (19). Supporting this notion, real-time PCR has revealed that expression of *insulin*



C

	JS-1	2.7A	3'DA	AFN-A	AFN-B	AFN-C	AFC
KO 1	0.052	0.196	0.081	0.053	0.435	0.088	0.074
2	0.620	0.171	0.034	0.043	0.227	0.076	0.066
3	0.084	0.094	0.050	0.099	0.089	0.051	0.167
4	0.186	0.058	0.058	0.093	0.187	0.047	0.213
5	0.043	0.057	0.037	0.059	0.103	0.105	0.110
6	0.028	0.027	0.057	0.023	0.055	0.045	0.081
7	0.339	0.095	0.035	0.050	0.082	0.207	0.072
8	0.178	0.034	0.034	0.071	0.062	0.044	0.076
9	0.084	0.178	0.037	0.079	0.259	0.054	0.307
10	0.053	0.083	0.865	0.020	0.136	0.038	0.796
11	0.264	0.060	0.046	0.151	0.103	0.186	0.075

FIGURE 3. Production of auto-Abs against α-fodrin in Aire-deficient mice. *A*, Schematic representation of α-fodrin. Black arrows and a blue arrow show the sites of cleavage by caspase 3 and calpain, respectively. *B*, Western blot analysis for recombinant α-fodrin with Aire-deficient mouse sera. Representative results from two mice from both wild-type and Aire-deficient mice are shown. Serum from NFS/*sld* mutant 3d-Tx mice reacted predominantly with the JS-1 fragment. 1, JS-1; 2, 2.7A; 3, 3′DA. *C*, Detection of auto-Abs against various forms of α-fodrin in sera from Aire-deficient mice using ELISA. Absorbance values greater than the mean ± 3 SD in wild-type mouse sera were considered positive and are colored.

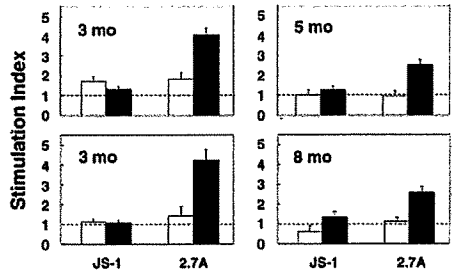


FIGURE 4. Autoreactive responses against α-fodrin by splenocytes from Aire-deficient mice. Proliferative responses of total splenocytes against two forms of recombinant α-fodrin (shown in Fig. 3*A*) were determined, and stimulation indices are demonstrated from control mice (open bars) and Aire-deficient mice (filled bars). Ages of the mice used are indicated.

and *salivary protein 1* was significantly reduced in the Aire-deficient thymic stroma (Fig. 5*A*). Because Aire-deficient mice developed autoimmunity against the defined target Ag, α-fodrin, we examined whether the expression of α-fodrin mRNA in the thymic stroma is changed in Aire-deficient mice. Using real-time PCR together with semiquantitative RT-PCR with three sets of primers encompassing the entire coding region of *α-fodrin*, we detected unrepressed *α-fodrin* expression from Aire-deficient thymic stroma when compared with that from control thymic stroma (Fig. 5, *A* and *B*); this was observed under the condition where the expression of *Foxn1*, which encodes a transcription factor involved in thymus development (39), was indistinguishable between the samples (Fig. 5*A*). Thus, our results suggest that Aire regulates self-tolerance beyond the transcriptional control of self-protein expression in the thymus, at least against this ubiquitously expressed protein.

To test whether autoreactivity against α-fodrin is associated with the development of inflammatory lesions in exocrine organs from Aire-deficient mice, we performed Western blot analysis using proteins extracted from the lacrimal glands. Both lacrimal glands and thymus from younger Aire-deficient mice (i.e., 3 mo) contained larger quantities of intact form α-fodrin (240 kDa) than the cleaved form (150 kDa), as observed for proteins from the control mice (Fig. 6*A*); this was demonstrated with two different kinds of Abs recognizing the C-terminal half (anti-α-fodrin mAb) and N-terminal half (anti-AFN-A polyclonal Ab) of α-fodrin. However, lacrimal glands from some aged Aire-deficient mice (i.e., 8 mo) contained a reduced amount of the intact form (Fig. 6*B*), although no detectable changes in α-fodrin expression in the thymus were observed in either form or quantity. This result suggests that autoreactivity against α-fodrin is associated with the pathogenetic process responsible for destruction of the lacrimal glands in this SS-like model, as observed in 3d-Tx-SS model (27, 38).

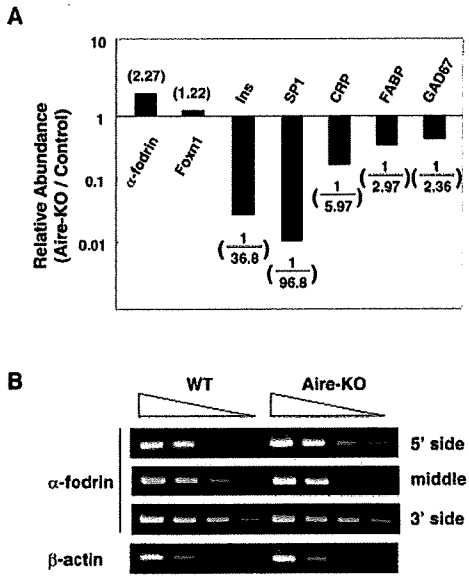


FIGURE 5. Unrepressed target Ag expression from Aire-deficient thymus. *A*, Real-time PCR for *α-fodrin*, *Foxn1*, and peripheral tissue-specific genes (i.e., Ins, *insulin*; SP1, *salivary protein 1*; CRP, *C-reactive protein*; FABP, *fatty acid-binding protein*; GAD67, *glutamic acid decarboxylase 67*) was performed using thymic-stroma RNAs from control and Aire-deficient mice. *Hprt* expression level was used as an internal control. Relative abundance of each gene was calculated from the ratio between the values from control thymus and those from Aire-deficient thymus (e.g., *insulin/Hprt* value from Aire-deficient mice was divided by *insulin/Hprt* value from control mice) and is shown in parentheses. One representative result from a total of three repeats is shown. *B*, Semiquantitative RT-PCR for *α-fodrin* was performed using thymic-stroma RNAs from control and Aire-deficient mice. *β-Actin* was used to verify equal amounts of RNAs in each sample. Three sets of primers encompassing the entire coding region of *α-fodrin* were used for detection. One representative result from a total of three repeats is shown.

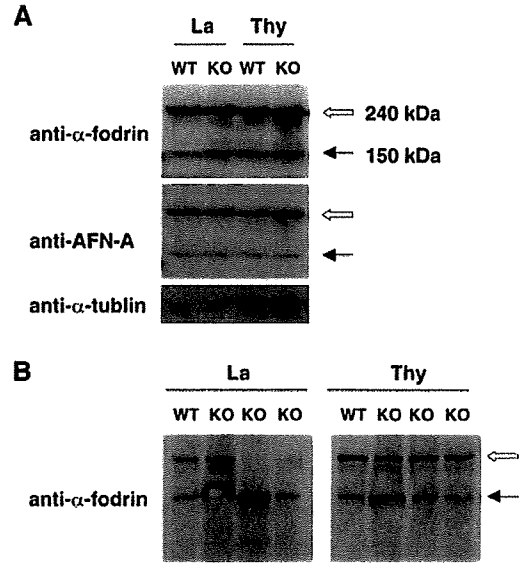


FIGURE 6. Autoreactivity against α-fodrin is associated with the pathogenetic process responsible for destruction of the lacrimal glands. *A*, Proteins extracted from the lacrimal glands and thymus of 3-mo-old mice were subjected to Western blot analysis using two different kinds of Abs recognizing the C-terminal half (anti-α-fodrin Ab, *top*) and N-terminal half (anti-AFN-A Ab, *center*) of α-fodrin. Open and filled arrows indicate the 240-kDa intact form and 150-kDa cleaved form of α-fodrin, respectively. The same blot was probed with anti-α-tubulin Ab (*bottom*). La, lacrimal gland; Thy, thymus. *B*, Proteins were extracted from the lacrimal glands and thymus of 8-mo-old mice. Western blot analysis was performed as shown in *A*. Lacrimal glands from some of the Aire-deficient mice showed a markedly reduced amount of the intact form (*left panel*, third and fourth lanes), although Aire-deficient thymus showed no detectable changes in α-fodrin expression in terms of form or quantity compared with control thymus (*right panel*). Open and filled arrows indicate the 240-kDa intact form and 150-kDa cleaved form of α-fodrin, respectively.

Loss of Aire in the thymic stroma is responsible for the breakdown of self-tolerance

Despite the predominant Aire expression in TECs, thymic structure was not apparently affected by the absence of Aire. Results of H&E staining as well as immunohistochemistry with the lectin *Ulex europaeus* agglutinin 1 (40) and ER-TR5 mAb (41), both recognizing a subset of mTEC, were indistinguishable between control and Aire-deficient mice (F. Kajiura, T. Ueno, Y. Takahama, and M. Matsumoto, unpublished observation). Organization of dendritic cells in the thymus identified with the mAb CD11c was also unaffected by Aire deficiency. Thus, Aire may not affect thymic organogenesis. Alternatively, relatively low frequencies of Aire-expressing cells among mTECs may account for the apparently normal thymic structure in Aire-deficient mice.

To investigate the impact of Aire deficiency in the thymic microenvironment, we generated thymic chimeras. Thymic lobes were isolated from control and Aire-deficient embryos of mixed background (H-$2^{b/k}$ × H-2^{b}) and cultured for 4 days in the presence of 2′-deoxyguanosine to eliminate thymocytes. Such thymic lobes did not contain any live thymocytes, as determined by flow cytometric analysis and Western blot analysis with anti-lck Ab (33). The lobes were then grafted under the renal capsule of BALB/c *nude* mice (H-2^{d}). Grafting of both control and Aire-deficient embryonic thymus induced T cell maturation in BALB/c *nude* mice at the periphery to a similar extent: $CD4^+$ T cells plus $CD8^+$ T cells were 12.5 ± 2.2% in *nude* mice grafted with control thymus (n = 6), compared with 12.3 ± 1.6% in *nude* mice grafted with Aire-deficient thymus (n = 7). It is important to note that the mature T cells produced de novo in both cases originated from Aire-sufficient *nude* mouse bone marrow (BM). Remarkably, histological examination of Aire-deficient thymus-grafted mice revealed infiltration of many lymphoid cells in the liver (mainly in the portal area) and pancreas (interlobular periductal and perivascular areas near islets) (Fig. 7, *A* and *B*). In contrast, we observed few such changes in control thymus-grafted mice.

To confirm that T cells developing in a thymic microenvironment without Aire are autoreactive per se, we injected splenocytes obtained from BALB/c *nude* mice grafted with Aire-deficient thymus into another group of BALB/c *nude* mice. We observed similar lymphoid cell infiltration in the liver of the recipient mice, whereas injection of splenocytes obtained from *nude* mice grafted with control thymus induced no such changes in the recipient mice (Fig. 7*B*). These results clearly indicate the significance of Aire as a thymic stromal element required for the establishment of self-tolerance.

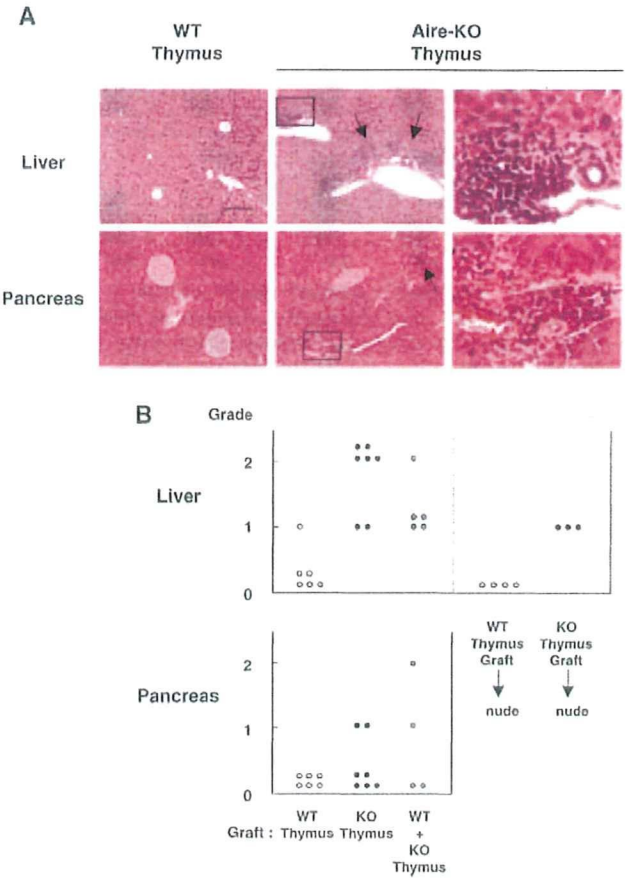


FIGURE 7. Thymic stromal elements in Aire-deficient mice are responsible for the development of autoimmunity. *A*, BALB/c *nude* mice grafted with Aire-deficient embryonic thymus (*middle panels*), but not with control embryonic thymus (*left panels*), developed an autoimmune disease phenotype in the liver and pancreas. The indicated areas are magnified in the *right panels*. Arrows indicate lymphoid cell infiltration. The scale bar corresponds to 100 μm. *B*, Many Aire-deficient thymus-grafted BALB/c *nude* mice exhibited lymphoid cell infiltration in the liver (*top*) and pancreas (*bottom*). In contrast, such changes were scarcely observed in mice grafted with control thymus. BALB/c *nude* mice grafted with both Aire-deficient thymus and control thymus showed significant pathological changes. Injection of splenocytes from BALB/c *nude* mice grafted with Aire-deficient thymus, but not with control thymus, into another group of BALB/c *nude* mice induced lymphoid cell infiltration in the liver of the recipient mice. Histological changes in H&E-stained tissue sections were scored as shown in Fig. 2*B*. One mark corresponds to one mouse analyzed.

Impaired regulation of autoreactivity in the absence of Aire

There is accumulating evidence that T cell-mediated dominant control of autoreactive T cells represents an important mechanism for the maintenance of immunologic self-tolerance (16, 17). We investigated whether loss of Aire in the thymus has a major impact on the production and/or function of Tregs. Spleen and thymus from adult Aire-deficient mice contained similar percentages as well as total numbers of $CD4^+CD25^+$ T cells compared with those from control mice (Fig. 8*A*). Real-time PCR for quantification of *Foxp3* mRNA (34, 42, 43) did not show any reduction of Tregs in the spleen of Aire-deficient mice (Fig. 8*B*). Expression of *Foxp3* in the whole thymus was also comparable between control mice and Aire-deficient mice (*Foxp3/Hprt* from wild-type mice = 1.8 vs *Foxp3/Hprt* from Aire-deficient mice = 2.4).

Recently, it has been demonstrated that functional alterations of Tregs could contribute to the development of autoimmune disease. A significant decrease in the effector function of $CD4^+CD25^+$ T cells from peripheral blood of patients with multiple sclerosis has been reported (44). It is of particular interest that the suppressor function of $CD4^+CD25^+$ T cells has been demonstrated to be defective in patients with autoimmune polyglandular syndrome type II, which is phenotypically closely related to APECED (also called autoimmune polyglandular syndrome type I) but whose pathogenesis is currently unknown (45). It is therefore important to test the function of Tregs from Aire-deficient mice. $CD4^+CD25^+$ T cells isolated from Aire-deficient mice dose-dependently suppressed [^{3}H]thymidine uptake by naive T cells cocultured in vitro with an efficiency nearly identical to that of $CD4^+CD25^+$ cells from control mice (Fig. 8*Ca*). This was also the case when responder cells ($CD4^+CD25^-$ cells) isolated from Aire-deficient mice were used for the assay (Fig. 8*Cb*). Thus, Aire does not have a major impact on the production and/or function of Tregs, at least as assessed in those assays.

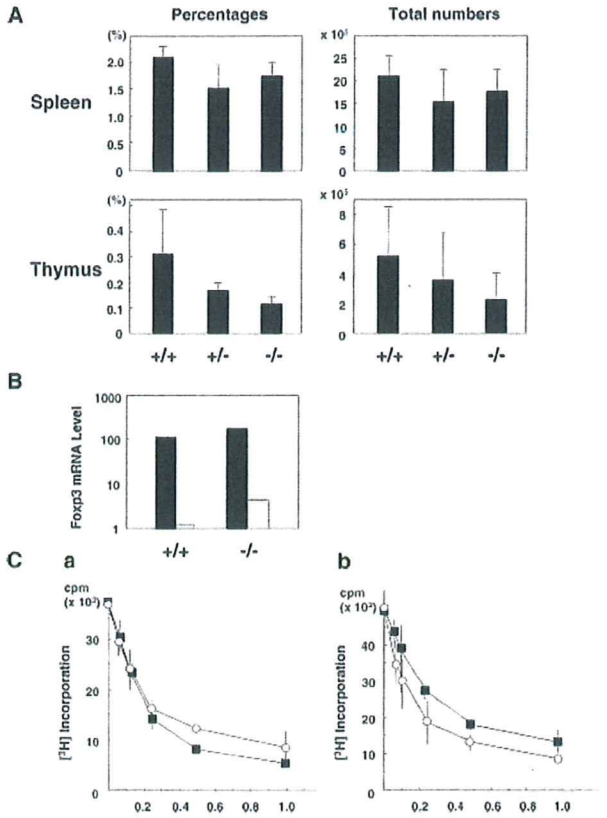


FIGURE 8. Retained production and function of Tregs from Aire-deficient mice. *A*, Spleens and thymuses from Aire-deficient mice contained percentages as well as total numbers of $CD4^+CD25^+$ T cells indistinguishable from those of control mice. $n = 5$, not statistically significant. *B*, Real-time PCR for *Foxp3* expression was performed using RNAs extracted from purified $CD4^+CD25^+$ (filled bars) and $CD4^+CD25^-$ T cells (open bars) with *Hprt* expression level as an internal control for the assay. One representative result from a total of two repeats is shown. *C*, $CD4^+CD25^+$ T cells isolated from Aire-deficient mice (*a*, ■) dose-dependently suppressed [^{3}H]thymidine uptake by native T cells from wild-type mice cocultured in vitro with an efficiency nearly identical to that of $CD4^+CD25^+$ cells from control mice (○). $CD4^+CD25^-$ T cells (2.5×10^4) were mixed with $CD4^+CD25^+$ T cells in various ratios as indicated on the *x*-axis. $CD4^+CD25^-$ T cells (2.5×10^4) were isolated from Aire-deficient mice (*b*), and their suppressive function was examined as shown in *a*. One representative result from a total of two repeats is shown.

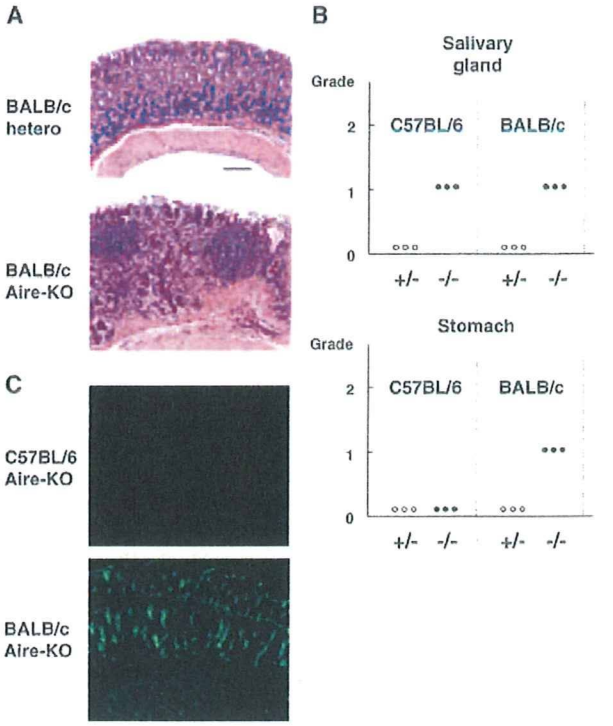


FIGURE 9. Strain-dependent target-organ specificity of the autoimmune disease caused by Aire deficiency. *A*, Aire-deficient BALB/c mice demonstrated lymphoid cell infiltration in the gastric mucosa (*bottom*). A scale bar corresponds to 100 μm in size (*top*; heterozygous Aire-deficient BALB/c mice). *B*, Aire-deficient BALB/c mice, but not Aire-deficient C57BL/6 mice, developed gastritis (*bottom*), whereas pathologic changes in the salivary glands were similarly observed in both strains (*top*). Histological changes in H&E-stained tissue sections were scored as shown in Fig. 2*B*. *C*, Aire-deficient BALB/c mice, but not Aire-deficient C57BL/6 mice, produced auto-Abs against gastric mucosa. Original magnification, ×100.

To gain further insight into how Aire contributes to the establishment of self-tolerance, we grafted control (Aire sufficient) and Aire-deficient embryonic thymus simultaneously into BALB/c *nude* mice. Inflammatory changes in the liver and pancreas of these animals were still present (Fig. 7*B*), supporting the hypothesis that impaired dominant control of autoreactive T cells by Tregs may not be the major defect caused by a thymic stroma lacking Aire; if impaired production of Tregs were the major defect caused by a thymic stroma lacking Aire, we assume that the defect should have been corrected by the grafted Aire-sufficient thymus. Therefore, it is reasonable to speculate that overproduction of autoreactive T cells plays an important role in the disease process triggered by Aire deficiency.

Strain-dependent target-organ specificity of the autoimmune disease caused by Aire deficiency

Although APECED is a monogenic disorder, it has been postulated that there may be additional factor(s) that determine the clinical features of the disease, such as the spectrum of affected organs (5, 6, 22). To test this hypothesis, we backcrossed our original strain of Aire-deficient mice to either the C57BL/6 ($H\text{-}2^b$) or BALB/c ($H\text{-}2^d$) strain for six generations. Both backcrossed strains showed autoimmune phenotypes similar to those from an original strain of Aire-deficient mice of mixed background (i.e., infiltration of many lymphoid cells in the salivary glands) (Fig. 9*B*, *top*). However, Aire-deficient BALB/c mice additionally demonstrated lymphoid cell infiltration in the gastric mucosa (Fig. 9, *A* and *B*, *bottom*), a feature that has been observed only rarely in the original Aire-deficient mice of mixed background (1 of 10) or Aire-deficient C57BL/6 mice (Fig. 9*B*, *bottom*). Consistent with these histological findings, serum harvested from Aire-deficient BALB/c mice (4 of 4) demonstrated strong auto-Abs against gastric mucosa (Fig. 9*C*), whereas this activity was observed in only one of four Aire-deficient C57BL/6 mice, and it was weak. Thus, the genetic background of the mice clearly influences the target-organ specificity of the disease caused by Aire deficiency.

Discussion

Using gene-targeted mice, we have investigated the mechanisms controlling the establishment and maintenance of self-tolerance by Aire. Both the numbers and suppressive function of $CD4^+CD25^+$ Tregs were not changed in Aire-deficient mice, when assessed in the adult mice. Using thymic chimeras, we also investigated possible defects in the production of any cell types (including

$CD4^+CD25^+$ Tregs) that are involved in the prevention of T cell-mediated organ-specific autoimmune diseases in the absence of Aire. When Aire-deficient and Aire-sufficient thymus were grafted simultaneously into *nude* mice, the development of inflammatory lesions was not completely inhibited. These results suggest that impaired production of Tregs may not be the major mechanism responsible for the breakdown of self-tolerance in Aire-deficient mice, and it is reasonable to speculate that the Aire-deficient thymus allows production of more pathogenic autoreactive T cells than could be controlled by the Tregs. However, it is important to emphasize that other aspects of Tregs, such as their repertoire formation, still remain unsolved; we cannot rule out the possibility that Aire may affect the Ag specificity of the Treg repertoire, because most of the analysis of the Tregs in the present study was quantitative rather than qualitative.

We have demonstrated that anti-α-fodrin autoimmunity developed in Aire-deficient mice despite the fact that the transcription of corresponding Ag (i.e., α-fodrin) in the thymic stroma was not down-regulated. Based on this finding, we suggest that Aire may regulate the processing and/or presentation of self-Ags by TECs, possibly through a coordinated action with BM-derived cells (see below), so that the maturing T cells can recognize the corresponding self-Ags in a form capable of efficiently triggering autoreactive T cells. It would be important to know whether our proposed model of Aire function in the establishment of self-tolerance is confined to ubiquitous self-Ags, such as α-fodrin, or applicable to tissue-specific Ags as well. In this regard, it is critical to investigate first whether autoimmunity develops bona fide against transcriptionally repressed tissue-specific Ags in the thymus in Aire-deficient mice. Definitively, identification of the substrate(s) for E3 ubiquitin ligase activity by AIRE should help to clarify the actual mechanisms of AIRE-dependent tolerance (10).

We have demonstrated that α-fodrin is one of the target Ags involved in the autoimmune-disease process caused by Aire deficiency. Because transfer of sera from affected mice did not result in the development of sialoadenitis or disruption of α-fodrin in the recipient mice (N. Ishimaru, R. Arakaki, and Y. Hayashi, unpublished observation), the disease process in Aire-deficient mice is most likely elicited by a cell-mediated immunity, as observed in the 3d-Tx-SS model (29, 30). Consistent with this hypothesis, splenocytes from Aire-deficient mice demonstrated proliferative responses in vitro when cultured with recombinant α-fodrin (Fig. 4).

Reduction of the intact form of α-fodrin in the affected lacrimal glands of some aged Aire-deficient mice (Fig. 6*B*) suggests that elicitation of autoreactivity against α-fodrin could be the primary pathogenetic process that leads to tissue destruction (27). In fact, adoptive transfer of α-fodrin-reactive T cells into ovariectomized B6 and SCID mice resulted in the development of autoimmune exocrinopathy quite similar to SS (30). However, based on the fact that α-fodrin is a ubiquitous protein and that the tissue destruction is confined to exocrine organs, it is reasonable to speculate that other undetermined tissue-specific target Ag(s) in exocrine organs might be additionally involved in the tissue destruction. Identification of precise target Ags involved in the disease process in Aire-deficient mice should help unravel the molecular mechanisms by which loss of Aire contributes to disease development.

We have demonstrated Aire-dependent disease development using allogeneic thymic chimeras; autoimmune disease commences in BALB/c *nude* recipients ($H\text{-}2^d$) of Aire-deficient, but not of wild-type, thymic transplants from mice of original mixed background ($H\text{-}2^{b/k} \times H\text{-}2^b$) (Fig. 7). The roles of TECs vs BM-derived cells in T cell repertoire selection in allogeneic thymic chimeras have been an issue of long-standing interest and debate. Given that *nude* mice reconstituted with an MHC-incompatible thymus generate effector T cells that are specific for the host and not for the thymic MHC (46), a novel mechanism may be responsible for the Aire-dependent negative selection; Aire expressed on TECs acts on BM-derived cells "in trans" as an important factor in organizing the "negative selection niche" in the thymus (47). This scenario is in good accordance with our results demonstrating the impaired tolerance to a ubiquitously expressed auto-Ag (i.e., α-fodrin) in Aire-deficient mice, because tolerance to ubiquitous self-proteins is mediated mainly by BM-derived cells in the thymus (48). Further study is required to test this intriguing hypothesis.

There is increasing evidence for the genetic complexity that underlies monogenic diseases (49, 50). In fact, the spectrum of the APECED phenotype is broad; the number of symptoms as well as the onset of each manifestation varies among affected patients. In our backcrossed mice, gastritis was observed predominantly in the BALB/c strain. In light of the fact that the individual HLA class II alleles modify the APECED phenotype (22), it is possible to speculate that MHC could be a candidate for the factor that determines this target-organ specificity. However, a genetic study with congenic strains has demonstrated that BALB/c ($H\text{-}2^d$), BALB.B ($H\text{-}2^b$), and BALB.K ($H\text{-}2^k$) were all susceptible to experimentally induced gastritis, whereas B10.D2 ($H\text{-}2^d$) were resistant, suggesting the predominant role of non-MHC gene(s) in determining susceptibility to autoimmune gastritis (51). Thus, MHC genes as well as non-MHC genes may together contribute to the complex phenotypes of APECED.

In conclusion, integration of detailed phenotypic analyses of Aire-deficient mice with current perspectives of thymus biology promises to illuminate many aspects of the molecular mechanisms responsible for the establishment and maintenance of self-tolerance. With the production of inbred strains of Aire-deficient mice, it may also be feasible to assess the impact of environmental factors that could influence the clinical features of APECED.

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Critical role of cathepsin-inhibitors for autoantigen processing and autoimmunity

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Introduction

The cysteinal lysosomal proteases, cathepsin S and cathepsin L have been shown to process invariant chain (Ii), thereby facilitating MHC class II maturation. However, their role in autoantigen processing is not established. Sjögren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La (Fox et al., 1986; Chan et al., 1991; Kruize et al., 1995). Previously, we reported a 120 kD α-fodrin autoantigen on the development of autoimmune exocrinopathy in SS model mice and identified autoantigen-specific T cell responses associated with Th1 cytokine production of interleukin (IL)-2 and interferon (IFN)-γ (Haneji et al., 1994, 1997). Insulin-dependent diabetes mellitus (IDDM) is a T-cell-mediated, organ-specific autoimmune disease that occurs in humans and in animal models such as the non-obese diabetic (NOD) mouse (Katz et al., 1993; Haskins and McDuffie, 1990). The autoimmune diabetes in NOD mice is characterized by lymphocytic infiltration of the islets (insulitis) followed by destruction of islet β-cells. Evidence for the role of $CD4^+$ Th1 cells in IDDM derives from recent studies of the NOD mouse in which the identical glutamic acid decarboxylase (GAD) and proinsulin are key β-cell autoantigens recognized by both T cells and B cells (Kaufman et al., 1993; Liu et al., 2002).

MHC class II molecules encounter and bind antigenic peptides as class II–peptide complexes on the cell surface of antigen-presenting cells (APCs) for recognition by $CD4^+$ T cells (Cresswell 1994; Gremain, 1994; Wolf and Ploegh, 1995). The molecular mechanisms leading to formation of class II–peptide complexes and

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