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, Fullerton, CA) as described previously (37). The purity of the $CD25^-$ and $CD25^+CD4^+$ populations was more than 90% and 95%, respectively. Spleen cells sorted as described above were co-cultured 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, Ontario, Canada) 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-like pathologic changes in exocrine organs from Aire-deficient mice

In order 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 mice (Figs. 1*B* and 1*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crossed with wild-type mice, homozygous crossing produced offspring only occasionally (F. Kajiura and M. Matsumoto, unpublished observation).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from the T cells stimulated with anti-CD3 mAb, were also unchanged by the Aire deficiency (S. Sun and M. Matsumoto, unpublished observation).

In order 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-$2^{b/k}$ x H-2^{b}). The most marked changes were evident in the lacrimal glands (Figs. 2*A* and 2*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 out of 8 Aire-deficient mice) and submandibular glands (10 out of 16 Aire-deficient mice) (Fig. 2*A*). Consistent with these Sjögren's syndrome (SS)-like pathologic changes in exocrine organs from Aire-deficient mice, secretion of tears per unit 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 out 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 months.

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 (Figs. 3*A* and 3*B*). Sera from 3d-Tx mice showed reactivity predominantly against the JS-1 fragment (27). Four out 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 out of eleven 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* 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 semi-quantitative 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 (Figs. 5*A* and 5*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.

In order 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 monoclonal Ab) and N-terminal half of α-fodrin (anti-AFN-A polyclonal Ab). 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).

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 (UEA-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 DCs 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.

In order 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-$2^{b/k}$ x H-2^{b}) and cultured for 4 days in the presence of 2'-deoxyguanosine in order 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 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) (Figs. 7*A* and 7*B*). In contrast, we observed few such changes in control thymus-grafted mice.

In order 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.

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 (APS) type II, which is phenotypically closely related to APECED (also called APS 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 native T cells co-cultured *in vitro* with an efficiency nearly identical to that of $CD4^+CD25^+$ cells from control mice (Fig. 8*C*, a). This was also the case when responder cells ($CD4^+CD25^-$ cells) isolated from Aire-deficient mice were used for the assay (Fig. 8*C*, b). Thus, Aire does not have a major impact on the production and/or function of Tregs, at least as assessed in those assays.

In order 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). In order to test this hypothesis, we backcrossed our original strain of Aire-deficient mice to either the C57BL/6 (H-2^b) or BALB/c (H-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). Aire-deficient BALB/c mice, however, additionally demonstrated lymphoid cell infiltration in the gastric mucosa (Figs. 9*A* and 9*B*, bottom), a feature that has been observed only rarely in the original Aire-deficient mice of mixed background (1 out 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 out 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 only 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. Employing 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 strom 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 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-2^d) of Aire-deficient, but not of wild-type, thymic transplants from mice of original mixed background (H-$2^{b/k}$ x H-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 the 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-2^d), BALB.B (H-2^b) and BALB.K (H-2^k) were all susceptible to experimentally induced gastritis, whereas B10.D2 (H-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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Figure Legends

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 probe A 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.

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, x100.

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.

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 mice thymectomized 3 days after birth (3d-Tx) 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 S.D. 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 (white bars) and Aire-deficient mice (black bars). Ages of the mice used are