ORIGINAL

Target-organ specificity of autoimmunity is modified by thymic stroma and bone marrow-derived cells

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Abstract : Physical contact between thymocytes and the thymic stroma is essential for the establishment of self-tolerance, and Aire in thymic epithelial cells plays an important role in this action. As expected, the autoimmune phenotypes of Aire-deficient mice are thymic stroma-dependent. Interestingly, the spectrum of the organs involved differs depending on the genetic background of non-autoimmune-prone mouse strains. Furthermore, deficiency of Aire in an autoimmune-prone strain of NOD also modifies target-cell specificity in the pancreas. In order to clarify the factors that regulate target-organ specificity in Aire-dependent autoimmunity, I have generated both thymic and bone-marrow chimeras, making it possible to evaluate the contribution of thymic stroma and bone-marrow-derived cells to this pathogenic process. The findings suggested that the genetic background of bone-marrow-derived cells contributes to the strain-dependent target-organ specificity of non-autoimmune-prone strains. Furthermore, in a study using NOD mice with a fixed genetic background, thymic stromal cells but not bone-marrow-derived cells were found to be relevant to the Aire-dependent alteration of target-cell specificity in the pancreas. These results clearly underscore the significance of immunological and/or genetic complexity that underlies Aire-deficiency monogenic disease together with critical dialogue between thymic stroma and bone-marrow-derived cells in the organized thymic microenvironment. J. Med. Invest. 54 : 54-64, February, 2007

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INTRODUCTION

Autoimmune disease is a pathological condition in which the immune system turns on itself and causes serious damage to host tissues through as yet unknown mechanisms (1). Breakdown of self-tolerance is considered to be the key event in initiating the disease process, and an understanding of the pathogenesis involved is crucial for developing a suitable therapeutic approach. Although it is now widely accepted that physical contact between thymocytes and the thymic stroma is essential for establishment of self-tolerance (2), the stromal elements that control these processes still remain elusive. Obviously, one of the critical roles of the thymic stroma in establishing self-tolerance is the elimination of pathogenic autoreactive T cells by negative selection (3). Thus, central tolerance is induced in the thymus, where developing thymocytes that recognize self-peptide-major histocompatibility complex (MHC) complexes with excessively high affinity are deleted ; T-cell development is a highly coordinated process that depends on interactions between thymocytes, thymic epithelium, and bone marrow (BM)-derived dendritic cells (DC). For decades, immunologists have tried to under-

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stand the roles of thymic epithelial cells (TEC) and BM-derived cells in this process (4). For negative selection, TECs, the key component of the thymic stroma, need to express a set of self-antigens (Ag) encompassing all the self-Ags expressed by parenchymal organs, a phenomenon termed promiscuous gene expression (PGE) (5, 6). Supporting this hypothesis, analysis of gene expression in the thymic stroma has demonstrated that epithelial cells of the medulla are a specialized cell type in which promiscuous expression of a broad range of tissue-restricted Ag (TRA) genes is an autonomous property (2, 5, 6).

Mutation of the autoimmune regulator (*AIRE*) gene is responsible for the development of an organ-specific autoimmune disease (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy ; APECED) that demonstrates monogenic autosomal recessive inheritance (7, 8). Because AIRE is predominantly expressed by TECs of the medulla (mTEC) (9), understanding the relationship between *AIRE* gene malfunction and the breakdown of self-tolerance promises to help unravel the pathogenesis of not only APECED, but also other types of autoimmune disease. As expected, deletion of the *Aire* gene in mice results in the development of organ-specific autoimmune disease, although there are differences in target-organ specificity between human patients and Aire-deficient mice (10-12). Interestingly, Aire-deficient TECs show reduced transcription of a group of genes encoding peripheral Ags (11-13). Based on this finding, it is reasonable to speculate that pathogenic autoreactive T cells escape negative selection because of reduced expression of the corresponding target Ags in the Aire-deficient thymus (11, 14). However, other mechanisms of Aire-dependent tolerance also remain possible. Indeed, subsequent studies have demonstrated that Aire-deficient mice develop autoimmunity against transcriptionally unrepressed target Ags in the thymus (12, 15, 16), and suggested that Aire might additionally regulate the processing and/or presentation of self-proteins so that maturing T cells can recognize self-Ags in a form capable of efficiently triggering autoreactive T cells.

An important aspect of the study of autoimmune disease is target-organ specificity. For example, β-cell islets are the predominant target of autoimmune attack in NOD mice, although the exact molecule(s) recognized by autoreactive T cells is still debatable (17). Similarly, autoimmune attack was found to be confined mostly to the exocrine organs, such as salivary and lacrimal glands, in our Aire-deficient mice. Interestingly, however, additional development of gastritis was observed in Aire-deficient mice of the BALB/c strain but not of the C57BL/6 (B6) strain (12). Thus, the genetic background of mice seems to predispose them to autoimmune diseases by influencing the pattern of the organs targeted and the disease severity. Furthermore, in Aire-deficient mice with a NOD background, acinar cells rather than β-cell islets are the major targets of autoimmune destruction (16), suggesting that Aire is also a strong modifier of target-organ specificity on a fixed genetic background, at least in NOD mice (18).

In order to gain further insights into the contribution of Aire to the establishment of central tolerance, as well as the pathogenic roles of Aire in regulating target-organ specificity, I have investigated how Aire-dependent target-organ specificity is affected by differences in the genetic background of mice. In this study, I have focused especially on the relationship between thymic stromal cells and BM-derived cells, such as DCs, in the control of target-organ specificity, because DCs in the thymus also play important roles in the establishment of self-tolerance (19). Results of thymic graft and BM transfer experiments demonstrated some of the unique features of Aire as well as the significance of cross-talk between the thymic stroma and BM-derived cells in the thymic microenvironment, which may help to clarify the cellular basis for the establishment of self-tolerance.

MATERIALS AND METHODS

Mice

C57BL/6J, BALB/c, athymic nude (*nu/nu*) mice on a C57BL/6J and BALB/c background, and NOD/Shi Jic mice were purchased from CLEA Japan, and Rag2-deficient mice on a BALB/c background were purchased from Taconic. Aire-deficient mice were generated as reported previously (12), and backcrossed onto either the B6, BALB/c (12) or NOD strain (16) for more than 6 to 8 generations. The mice were maintained under pathogen-free conditions, and handled in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine.

Thymus graft

Thymus grafting was performed as described previously (20). Briefly, thymic lobes were isolated

from embryos at 14.5 days postcoitus, and cultured for 4 days on Nuclepore filters (Whatman) placed on RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-ME, hereafter referred to as R10, containing 1.35 mM 2'-deoxyguanosine (2-DG) (Sigma-Aldrich). During the course of this culture, thymocytes which are sensitive to 2-DG are eliminated from the fetal thymus, leaving behind only the 2-DG-resistant stromal cells. Five pieces of thymic lobes were grafted under the renal capsule of nude mice. After 6 to 8 weeks, reconstitution of peripheral T cells was determined by flow-cytometric analysis (BD Bioscience) with anti-CD4 (clone GK1.5 ; BD PharMingen) and anti-CD8 (clone 53-6.7 ; BD Bioscience) monoclonal antibodies, and then thymic chimeras were used for analysis.

BM transfer

BM transfer was performed as described previously (21). In brief, BM cells were suspended in R 10 medium containing anti-CD90 (Thy 1.2) monoclonal antibody (clone 5a-8 ; Cedarlane Laboratories) plus low-toxicity rabbit complement (Cedarlane Laboratories) to eliminate T cells, thereby minimizing any graft versus host reactions. After incubation at 37 ℃ for 45 min, the cells were washed twice and adjusted to 3 x 10^7 viable cells/ml in R 10. Each recipient mouse was lethally irradiated (10 Gy) and treated with 0.5 ml of donor BM cells i.v. on the same day. The recipient mice were used for analysis 12 weeks after BM transfer.

Pathology

Formalin-fixed tissue sections were subjected to H&E staining. Histological changes were scored as 0 (no change), 1 (mild lymphoid cell infiltration) or 2 (marked lymphoid cell infiltration).

Detection of autoantibody

For detection of autoantibodies, mouse serum was incubated with various organs obtained from Rag2-deficient mice. FITC-conjugated anti-mouse IgG antibody (Southern Biotechnology Associates) was used for detection, and autoantibody titers were scored as 0 (negative), 1 (moderate) or 2 (strong). Scoring for both histological evaluations and detection of autoantibodies was performed in a blind manner.

Flow-cytometric analysis

Spleen cell suspensions were prepared by teasing the tissues apart between two frosted microscope slides. The suspensions were depleted of RBC by osmotic lysis, and the cells were stained with monoclonal antibodies. Anti-Foxp3 monoclonal antibody was from eBioscience. The cells were analyzed using a FACScaliber flow cytometer (BD Bioscience) with CELLQuest software, as described previously (21).

RESULTS

Pathological changes in thymic chimeras

Although APECED is a monogenic disorder, it has been postulated that there should be additional factor(s) that determine the clinical features of the disease, such as the spectrum of affected organs (7, 8, 22). Indeed, we found that Aire-deficient BALB/c mice demonstrated gastritis in addition to infiltration of many lymphoid cells in the salivary glands, a feature that has not been observed in Aire-deficient B6 mice (12). Although this result clearly indicated a strain-dependent target-organ specificity of the autoimmune disease caused by Aire deficiency, contribution of thymic stromal cells versus BM-derived cells on different genetic backgrounds to this pathologic process was not demonstrated. To investigate the impact of the strain-dependent thymic microenvironment that contributes to target-organ specificity, I generated thymic chimeras. 2-DG-treated embryonic thymic lobes either from Aire-deficient B6 mice or Aire-deficient BALB/c mice were prepared, and then grafted under the renal capsule of nude mice of either a B6 or BALB/c background. Thymus grafting of all the four combinations (i.e, B6 onto B6, BALB/c onto B6, B6 onto BALB/c, and BALB/c onto BALB/c) irrespective of the genotypes induced peripheral T cell maturation in recipient nude mice to a similar extent ; $CD4^+$ T cells plus $CD8^+$ T cells in the spleen from chimeric mice were 17.9 ± 3.0% in B6 Aire-sufficient thymus onto B6 ; 17.2 ± 3.0% in B6 Aire-deficient thymus onto B6 ; 12.7 ± 1.8% in BALB/c Aire-sufficient thymus onto B6 ; 18.5 ± 5.2% in BALB/c Aire-deficient thymus onto B6 ; 15.4 ± 4.8% in B6 Aire-sufficient thymus onto BALB/c ; 12.2 ± 1.6% in B6 Aire-deficient thymus onto BALB/c ; 12.9 ± 2.5% in BALB/c Aire-sufficient thymus onto BALB/c ; 13.3 ± 3.0% in BALB/c Aire-deficient thymus onto

BALB/c ; 2.7 ± 0.3% in untreated BALB/c nude mice. It is important to note that the mature T cells produced de novo, as well as the DCs in the thymus, originated from the recipient nude mouse BM.

As expected, grafting of wild-type B6 thymus onto either B6 or BALB/c nude mice produced no evident pathological changes in the liver, pancreas or stomach (Fig. 1A). In contrast, grafting of Aire-deficient B6 mouse thymus onto both B6 and BALB/c nude mice induced various degrees of pathological change in these organs ; lymphoid cell infiltration was observed mainly in the portal area of the liver, in the interlobular periductal and perivascular areas near pancreatic islets, and in the gastric mucosa (Figs. 1A and 1B). Stomach lesions of BALB/c nude mice grafted with Aire-deficient B6 mouse thymus were only slightly more severe than those in B6 nude mice grafted with the same Aire-deficient B6 mouse thymus (5 out of 6 versus 2 out of 4, all with grade 1). When BALB/c mice were used as the donor strain, even grafting of Aire-sufficient thymus induced some degrees of lymphoid cell infiltration in organs of both B6 and BALB/c nude mice, as we had observed previously (12). As expected, however, grafting of Aire-deficient BALB/c mouse thymus clearly produced more severe and frequent pathological changes in all organs compared with grafting of Aire-sufficient BALB/c mouse thymus. Strains of the recipient nude mice, irrespective of whether they had a B6 or BALB/c background, did not show any significant difference in the stomach lesions. Furthermore, grafting of Aire-deficient B6 mouse thymus into BALB/c nude mice resulted in stomach lesions similar in severity to those in B6 or BALB/c nude mice grafted with Aire-deficient BALB/c mouse thymus. Thus, although thymus graft experiments clearly demonstrated the impact of Aire in the thymic stroma, my attempt to specify the stain-dependent components (i.e., thymic stroma from the donor and/or BM-derived cells from the recipient) responsible for the development of BALB/c-dependent gastritis was not sufficiently achieved by histological evaluation alone.

Autoantibody production in thymic chimeras

Since it was clarified that histological evaluation of thymic chimeras alone does not necessarily indicate the components of the stomach lesions observed predominantly in untreated Aire-deficient mice of a BALB/c, but not a B6, background (and

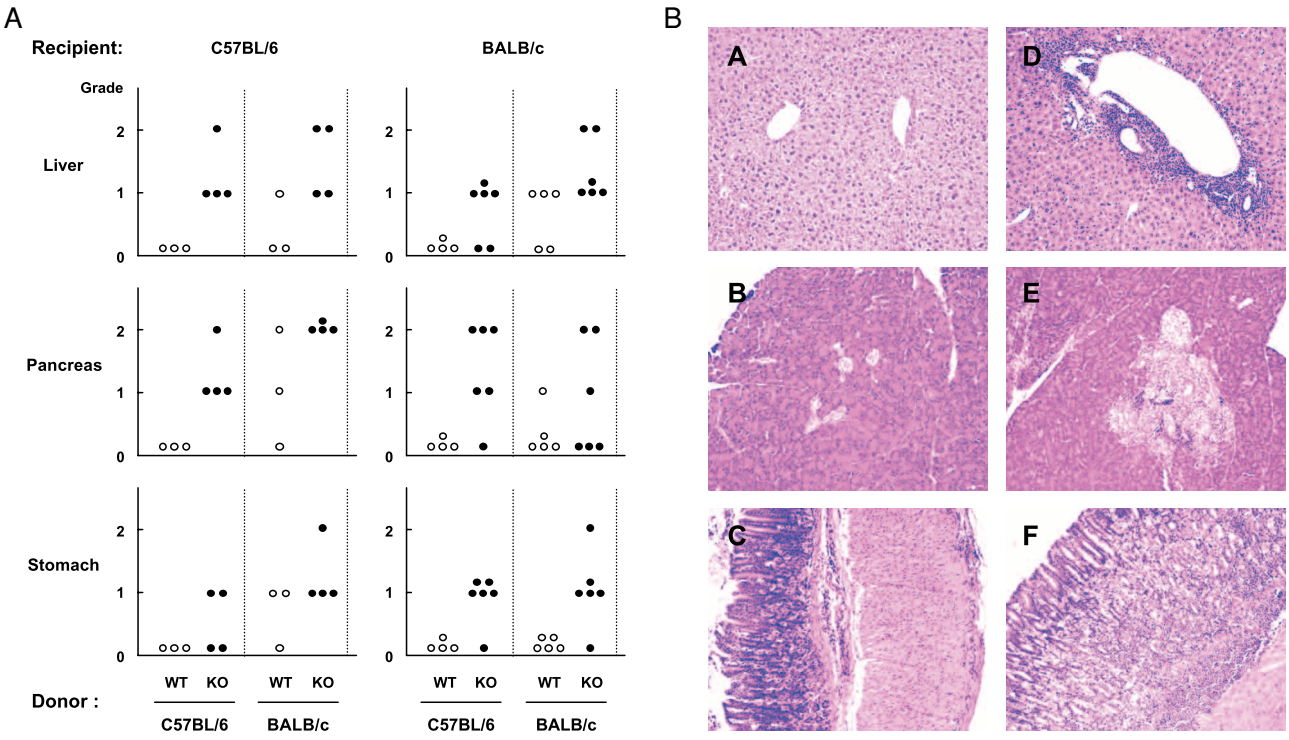

Fig. 1. Histological evaluation of pathological changes in organs from thymic chimeras.
A. Thymic chimeras were generated using donor and recipient mice with different combinations of genetic background. Histological changes in H&E-stained tissue sections were scored as 0 (none), 1 (moderate) or 2 (strong). One mark corresponds to one mouse analyzed.
B. Representative pathological changes in the liver (A and D), pancreas (B and E) and stomach (C and F) from thymic chimeras generated with Aire-deficient embryonic thymus (D to F). Such changes were scarcely observed in mice bearing Aire-sufficient embryonic thymus grafts (A to C). Original magnification, x100.

in fact, half of the B6 nude mice — even those with Aire-deficient B6 mouse thymus grafts — showed stomach lesions to some extent), I then investigated the target-organ specificity of autoantibodies produced in the chimeric animals. Indeed, we had previously observed the production of autoantibodies against gastric mucosa in Aire-deficient mice of a BALB/c, but not of a B6, background, supporting the finding of strain-dependent gastritis (12).

As observed for pathological changes, grafting of Aire-sufficient B6 thymus into either B6 or BALB/c nude mice resulted in no production of autoantibodies against liver, pancreas or stomach, except in two BALB/c nude mice : one against pancreas and the other against stomach, the titers being low in both cases (Fig. 2A). In contrast, grafting of Aire-deficient B6 mouse thymus into both B6 and BALB/c nude mice raised various degrees of autoantibody production against these organs (Figs. 2A and 2B). It is important to note that BALB/c nude mice with thymus grafts from Aire-deficient B6 mice showed much more frequent and higher titers of autoantibody production against stomach than B6 nude mice bearing the same grafts. Grafting of Aire-deficient BALB/c mouse thymus into B6 nude mice resulted in similar degrees of autoantibody production to those in B6 nude mice bearing thymus grafts from Aire-deficient B6 mice. When BALB/c nude mice were used as the recipients, grafting of Aire-sufficient thymus from mice of either a B6 or BALB/c background resulted in some degree of autoantibody production in several mice. BALB/c nude mice bearing thymus grafts from Aire-deficient mice of either a B6 or BALB/c background produced much higher titers of autoantibodies, especially against the stomach, than B6 nude mice bearing the same set of Aire-deficient mouse thymus grafts. However, the thymus donor strains, irrespective of whether the background was B6 or BALB/c, showed no significant difference in the magnitude, frequency or spectrum of autoantibody production, including that against the stomach. Thus, the recipient strain of mouse (which provides BM-derived cells including DCs in the thymus), rather than the donor strain of thymic stroma, appears to play a critical role in the stain-dependent target-organ specificity of antibody

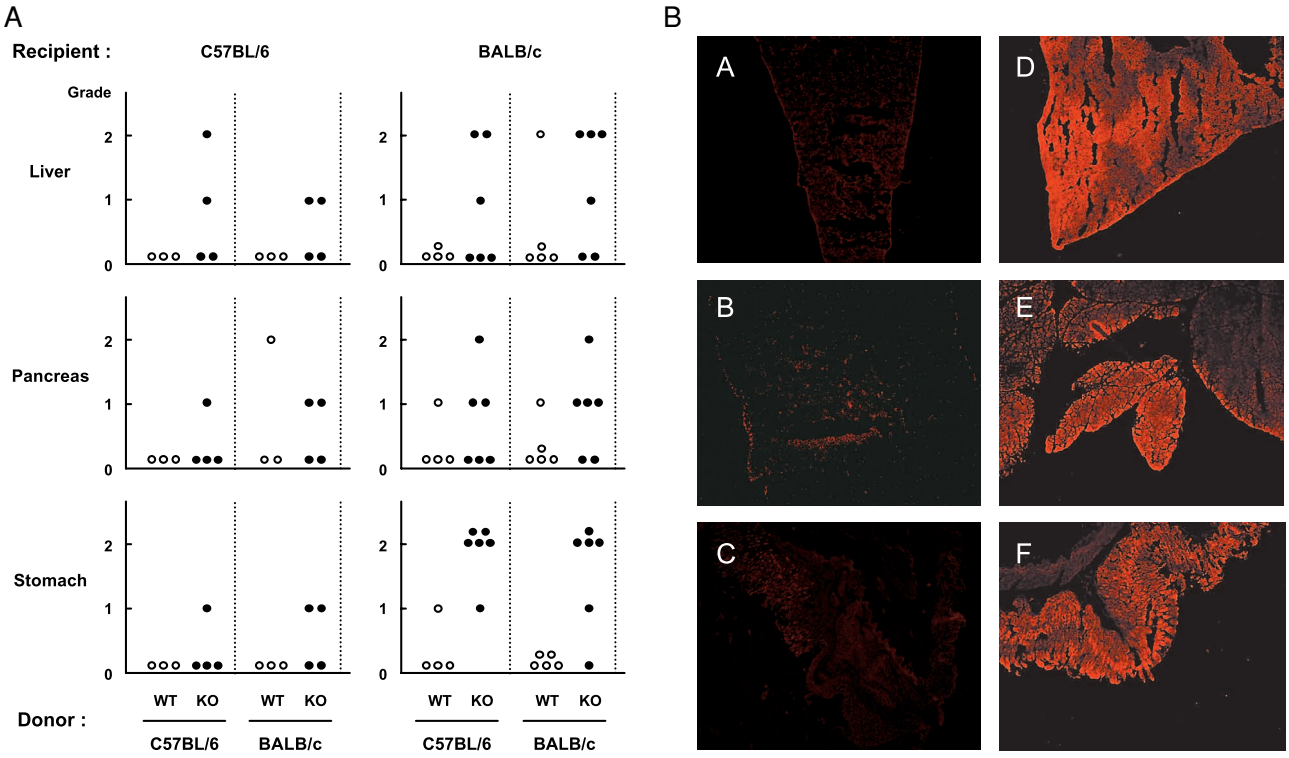

Fig. 2. Production of autoantibodies in thymic chimeras.
A. Autoantibody production against various organs detected by immunofluorescence was scored as 0 (none), 1 (moderate) or 2 (strong). High frequencies of autoantibodies against stomach tissue were observed when the recipient nude mice had a BALB/c background compared with when the recipients had a B6 background, irrespective of the donor strain. One mark corresponds to one mouse analyzed.
B. Serum from thymic chimeras generated with Aire-deficient embryonic thymus contained IgG class autoantibodies against liver (D), pancreas (E) and stomach (F) detected by immunofluorescence. Such autoantibody production was scarcely detected in mice bearing Aire-sufficient embryonic thymus grafts (A to C). The results from thymic chimeras with Aire-sufficient thymus (A to C) and Aire-deficient thymus (D to F) demonstrated were scored as 0 and 2, respectively. Original magnification, x40.

production caused by Aire deficiency.

Impact of Aire deficiency in thymic stroma on altered intra-pancreatic target-cell specificity in NOD mice lacking Aire

We have recently demonstrated that acinar cells rather than β-cell islets are the major targets of autoimmune destruction in Aire-deficient mice on a NOD background (16). However, it is not yet clear whether this Aire-dependent alteration of intra-pancreatic target-cell specificity is totally dependent on Aire in the thymic stroma. It is possible that BM-derived cells might also be relevant in this respect, as suggested by the results described above. Consistent with this idea, it has been suggested that Aire in DCs is involved in the establishment of self-tolerance (23). In order to investigate this issue, I have generated thymic chimeras using Aire-deficient NOD mice as donors and BALB/c nude mice as recipients, and examined the pathological changes as well as the autoantibody production in these animals (Table 1). Grafting of Aire-sufficient NOD mouse thymus induced lymphoid cell infiltration in the liver, pancreas and stomach to some degree (Fig. 3A). Notably, production of autoantibodies in these mice was more profound than that resulting from grafting of Aire-sufficient thymus from mice with a B6 or BALB/c background (compare with Fig. 2A), suggesting that the thymic stroma of NOD mice may have characteristics conducive to autoimmune induction. When thymus grafts from Aire-deficient NOD mice were used, all the recipient mice showed pathological changes in all organs examined (Fig. 3A). Remarkably, acinar cells rather than β-cell islets were the major targets of autoimmune destruction in those mice, as was the case in untreated Aire-deficient NOD mice (Table 1 and Fig. 3B). This result suggests that Aire-deficient thymic stroma largely accounts for the alteration of intra-pancreatic target-cell specificity observed in untreated Aire-dependent NOD mice. None of the mice examined showed signs of development of overt diabetes during the course of observation for up to 5 months (unpublished observation). There was no difference between thymic chimeras generated with Aire-sufficient thymus and Aire-deficient thymus in the amount of $CD4^+Foxp3^+$ immunoregulatory T cells (Treg) (24) in the spleen (Table 1).

No major contribution of Aire deficiency in BM-derived cells to altered intra-pancreatic target-cell specificity in NOD mice lacking Aire

The results obtained from the above experiments suggested that Aire in the thymic stroma plays a major role in the unique feature of autoimmune pathogenesis observed in NOD mice lacking Aire. In order to further confirm this finding, I performed a BM transfer experiment in which BM cells harvested from either Aire-deficient NOD mice or Aire-sufficient littermates were transferred to lethally irradiated Aire-wild-type NOD mice.

Table 1. Thymic chimeras generated with NOD embryonic thymus

Genotypes of donor mice	Histological changes in pancreas Grade/Target tissue	$CD4^+ + CD8^+$ (%)	$CD4^+Foxp3^+$ (%)
+/-	1 / islet	11.9	N.D.
+/-	0 / N.A.	21.9	3.21
+/-	0 / N.A.	25.0	3.56
+/-	0 / N.A.*	24.9	4.53
+/-	0 / N.A.	22.8	N.D.
		21.3 ± 5.4	3.77 ± 0.68
-/-	1 / acinus ≧ islet	18.2	3.11
-/-	1 / acinus >> islet	18.5	2.36
-/-	2 / acinus >> islet**	27.0	7.78
-/-	2 / acinus >> islet	17.3	N.D.
		20.3 ± 4.5	4.42 ± 2.94

N.A., not assessed.
N.D., not determined.
*, **, histology of these individual mice is shown in Fig. 3B.
Degrees of predominance of target-tissue destruction (i.e., islet or acinus) are expressed as ≧ or >>.

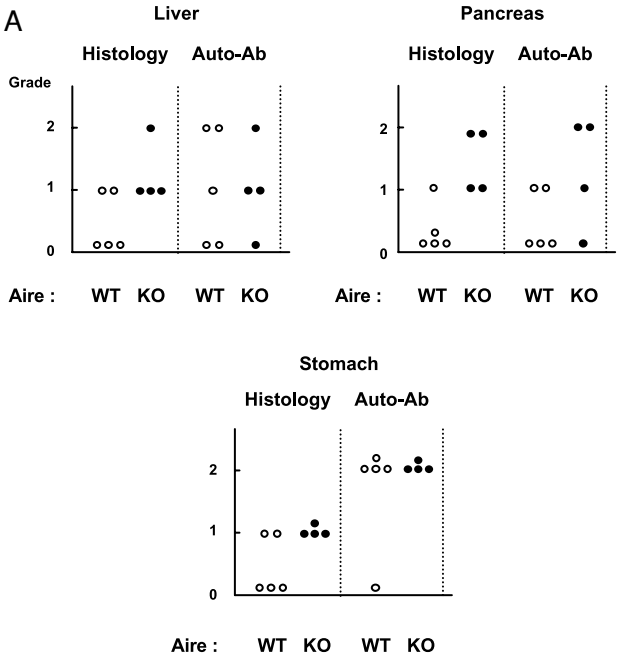

Fig. 3. Pathological changes in thymic chimeras generated with Aire-deficient NOD mouse thymus.
A. Histological changes and autoantibody production in thymic chimeras generated with Aire-sufficient or Aire-deficient NOD embryonic thymus were scored as in Figs. 1 and 2. One mark corresponds to one mouse analyzed.
B. Representative pathological changes in the pancreas from thymic chimeras generated with Aire-sufficient NOD embryonic thymus (A) or Aire-deficient NOD embryonic thymus (B). Original magnification, x100.

Histological examination of BM-chimeras revealed that NOD mice that had received both Aire-deficient NOD mouse BM and Aire-sufficient littermate BM (2 mice for each group) showed similar degrees of peri-insulitis in the pancreas, which is a common pathological change seen in wild-type NOD mice before the onset of overt diabetes ; no acinar cell destruction was observed in recipient mice that had received BM grafts from Aire-deficient NOD mice (data not shown). None of the mice had developed overt diabetes by 12 weeks after BM transfer. Thus, Aire in BM-derived cells plays little part, if any, in determining the unique Aire-dependent intra-pancreatic target-cell specificity observed in NOD mice from this limited numbers of the analysis.

DISCUSSION

In the present study, I examined the factors that modify the target-organ specificity of autoimmune disease seen in Aire-deficient mice with the use of two different models through the generation of thymic chimeras. The first model involved strain (BALB/c but not B6)-dependent development of gastritis (12), and the second involved Aire-dependent alteration of intra-pancreatic target-cell specificity in NOD mice (16). The former model suggested a role of BM-derived cells, whereas the latter suggested that BM-derived cells had little involvement. However, it is important to emphasize that these findings are never mutually exclusive. In the first model, mice with different combinations of genetic background were used as both thymus donors and recipients ; Ag-presenting cells such as DCs (i.e., BM-derived cells) originate from recipient mice harboring wild-type Aire, if expressed, irrespective of the strain. Therefore, only the difference in genetic background of both TECs and BM-derived cells is the factor that affects the outcome in this model of thymic-stroma-dependent autoimmunity caused by lack of Aire. In contrast, the latter model involved the use of thymic stroma from mice with a fixed NOD genetic background, as well as recipient nude mice with a fixed BALB/c genetic background possessing wild-type Aire in BM-derived cells, if expressed. Thus, the results obtained with the second model depended only on the presence or absence of Aire in mTECs. In this regard, it is also important to mention that the recipient nude mice of the BALB/c strain that provide the BM-derived cells are not autoimmune-prone, and this may have explained the failure to completely reconstitute the autoimmune pathogenesis in Aire-deficient NOD mice in the thymic chimeras I generated.

Although histological examination of thymic chimeras alone did not successfully indicate the population (thymic stroma versus BM-derived cells) that is critical for the strain-dependent development of gastritis in the first model, together with the evaluation of autoantibody production in these mice, it was evident that BM-derived cells contributed to this process. Given that BM-derived cells in the thymus can present self-Ags derived from mTECs to developing T cells (19), it is possible that the spectrum and/or doses of self-Ags presented by DCs in the thymus might differ between the B6 and BALB/c strains. For this scenario, I assume

that a strain-dependent negative selection niche in the thymic microenvironment might play a role in strain-dependent target-organ specificity. However, it is also possible that differences in the features of Th1 versus Th2 balance in peripheral effector T cells between mice of different genetic background (25) might account for the preferential development of gastritis in Aire-deficient BALB/c mice. Thus, although AIRE deficiency is a monogenic disease, it is essential to understand the immunological and/or genetic complexity that underlies the disorder, as this would yield important insights into the broad spectrum of the APECED phenotype (9).

I have utilized nude mice reconstituted with a fully allogeneic thymus graft for the assessment of the roles of thymic stroma versus BM-derived cells in determining target-organ specificity of autoimmune disease. Whether the autoreactive T cells developed in the hosts were donor (thymic stroma)-restricted or host (BM-derived)-restricted was not addressed in my study. It is widely accepted that the MHC of radio-resistant cells of the thymus (presumably TECs) selects the T cell repertoire based on a series of classical irradiation BM and thymus chimera experiments (26). Also, injection of allogeneic fibroblasts (27, 28) or TECs (29) into the thymus of mice and fetal thymus organ culture experiments (30) indicated positive selection of thymocytes restricted to the MHC of these cells. However, studies with allogeneic thymus graft, like employed in the present study, showed almost exclusive restriction to nude (BM-derived) MHC alone (31), although the studies were not accepted completely because of suspected rescue of nude thymic rudiment. This issue was later approached with the generation of tetraparental aggregation chimeras from thymus-deficient nude mice and Rag-deficient mice devoid of mature T and B cells, and suggested that MHC of non-TECs (BM-derived cells) efficiently selects a functional repertoire (32). More recently, tetraparental aggregation chimeras lacking MHC class II or both MHC I and II molecules on TECs, but not on cells of nude origin were generated, which furthermore demonstrated that chimeras with MHC-deficient TECs mounted functional virus-specific $CD8^+$ but not $CD4^+$ T cell responses. Thus, maturation of functional $CD4^+$ T cell responses require MHC class II on TECs, whereas $CD8^+$ T cells mature in the absence of MHC on TECs (33). In this regard, if autoreactive $CD8^+$ T cells were developed under the host-restriction in the thymic chimeras I have generated, it suggests an intriguing mechanism for the Aire-dependent T-cell repertoire (negative selection) formation ; Aire expressed on TECs act on BM-derived cells "in trans" as an important factor in organizing the "negative selection niche" in the thymus. Further experiments are required to approach this issue.

It is still unclear whether Aire itself in BM-derived cells, particularly DCs, is involved in the pathogenic process of autoimmunity. One report has demonstrated that Aire-deficient DCs activate naive T cells more efficiently than Aire-sufficient DCs, implying a role for Aire in peripheral DC regulation of T-cell activation (23). Furthermore, it has been demonstrated that AIRE expression is induced in human blood $CD11c^+$ DCs by thymic stromal lymphopoietin (TSLP) (34), although the functional significance of this for self-Ag expression and T-cell repertoire selection *in vivo* remains unknown. In contrast to reports on the roles of Aire in the periphery, experiments with BM transfer together with thymus grafting have suggested that Aire in the thymic stroma is necessary and sufficient for the induction of Aire-dependent autoimmunity ((11) and this study, at least for alteration of intra-pancreatic target-cell specificity in NOD mice). However, this does not imply that BM-derived cells in the thymus are not relevant to the establishment of self-tolerance, as already discussed above. Because both Aire-dependent and Aire-independent self-Ags expressed by thymic stromal cells could be transferred to the BM-derived cells for efficient elimination of autoreactive T cells (19), and this process might take place irrespective of the Aire expression by BM-derived cells, I assume that BM-derived cells in the thymus are the important components for self-Ag expression in the thymus beyond expression and/or the function of Aire in BM-derived cells, part of which was demonstrated by the present study. Definitively, more comprehensive studies focusing on the roles of BM-derived cells in the organization of thymic microenvironment are necessary in the future, for instance, with the use of BM cells defective for Ag presentation.

We and others have demonstrated that Aire has no major impact on the production and/or function of Tregs in both non-autoimmune-prone and autoimmune-prone strains of mice (12, 14-16). With the use of thymic chimeras, I also demonstrated that production of Tregs assessed by detection of Foxp3-expressing cells was not affected by absence of Aire in the thymic stroma (Table 1). Given that Tregs arise from relatively high-avidity inter-

actions with self-peptide-MHC complexes just below the threshold for negative selection (24, 35, 36), it is somewhat unexpected that Aire deficiency has no major impact on the production or suppressive function of Tregs. This issue, however, needs to be further investigated using different models of Treg-dependent autoimmunity, because most analyses of Tregs carried out so far have been quantitative rather than qualitative.

Although many studies have suggested that defective central tolerance in NOD mice is caused primarily by an intrinsic defect of apoptosis in NOD thymocytes during negative selection (37-39), one study has found that grafting of embryonic thymus from NOD mice into B6 nude mice resulted in the development of peri-insulitis and insulitis in approximately one third of the recipient mice, albeit without development of overt diabetes (40). In the present study, only one out of five thymic chimeras generated with Aire-wild-type NOD thymus developed mild insulitis, and none of them developed diabetes during the course of observation for up to 5 months (Table 1). However, I noticed a higher frequency of autoantibody production in these mice compared with the thymic chimeras generated using Aire-sufficient B6 or BALB/c thymus, suggesting that NOD mice have an intrinsic defect of the thymic stroma. A propensity of the NOD thymic stroma to autoimmunity was also suggested by exaggerated acinar cell destruction in the thymic chimeras, which was never evident in other thymic chimeras generated from Aire-deficient non-autoimmune-prone strains (Fig. 3B). The exact nature of the thymic stromal defect in NOD mice would be an interesting issue to pursue in future studies.

Finally, I would like to address a simple but fundamental question about the roles of Aire in the establishment of self-tolerance. Because initial studies suggested that Aire is a transcriptional regulator of self-Ag expression, it was hypothesized that Aire is required by particular type(s) of mTECs in order for them to exert their tolerogenic function. This model hypothesizes that Aire-positive cells are specialized cell type(s) that have unique ability to express a broad range of self-Ags (i.e., PGE). The model also assumes that mTECs eventually gain properties of PGE by becoming differentiated (terminal differentiation model) (13). One potential problem of this model is that it remains to be demonstrated whether Aire-positive cells are the major source of PGE from mTECs per se. In this regard, a recent single-cell analysis has demonstrated that, to the contrary, expression of Aire in a mTEC is not sufficient for simultaneous coexpression of Aire-dependent TRA genes (41). In contrast to the terminal differentiation model, the alternative model suggests that Aire is an important differentiation factor that determines the fate of immature mTECs (developmental model) (42). As a result, Aire expression does not have to guarantee the PGE, as described above (41). In the developmental model, expression of a broad spectrum of TRA genes is considered to be regulated by conserved developmental programs active in developing mTECs. Thus, in the near future, it will be necessary to clarify whether Aire is necessary within particular type(s) of mTECs (favoring the terminal differentiation model) or whether Aire-expressing cells are required for organization of the thymic microenvironment (favoring the developmental model). In order to do this, we will need to establish specific markers of cells other than those with Aire expression that would make it possible to monitor the developmental process of Aire-bearing cells.

In conclusion, I have demonstrated that Aire is not only critical for the establishment and maintenance of self-tolerance in the thymic stroma but also a strong modifier of the spectrum of autoimmune disease, as exemplified by the unique intrapancreatic target-cell specificity observed in thymic chimeras generated from the autoimmune-prone NOD mouse strain. Furthermore, strain-dependent target-organ specificity in Aire-deficient non-autoimmune-prone strains underscored the significance of BM-derived cells for the control of T-cell repertoire formation. Future studies will be needed to clarify the molecular mechanisms by which Aire contributes to these fundamental processes, hopefully leading to complete understanding of the self versus non-self discrimination mechanism of the immune system.

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REFERENCES

1. Kamradt T, Mitchison NA : Tolerance and autoimmunity. N Engl J Med 344 : 655-664, 2001
2. Kyewski B, Klein L : A central role for central tolerance. Annu Rev Immunol 24 : 571-606, 2006
3. Hogquist KA, Baldwin TA, Jameson SC : Central tolerance : learning self-control in the thymus. Nat Rev Immunol 5 : 772-782, 2005
4. Zinkernagel RM, Althage A : On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells. Proc Natl Acad Sci USA 96 : 8092-8097, 1999
5. Derbinski J, Schulte A, Kyewski B, Klein L : Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat Immunol 2 : 1032-1039, 2001
6. Kyewski B, Derbinski J, Gotter J, Klein L : Promiscuous gene expression and central T-cell tolerance : more than meets the eye. Trends Immunol 23 : 364-371, 2002
7. Björses P, Aaltonen J, Horelli-Kuitunen N, Yaspo ML, Peltonen L : Gene defect behind APECED : a new clue to autoimmunity. Hum Mol Genet 7 : 1547-1553, 1998
8. Pitkänen J, Peterson P : Autoimmune regulator : from loss of function to autoimmunity. Genes Immun 4 : 12-21, 2003
9. Peterson P, Peltonen L : Autoimmune polyendocrinopathy syndrome type 1 (APS1) and AIRE gene : new views on molecular basis of autoimmunity. J Autoimmun (Suppl 25) : 49-55, 2005
10. Ramsey C, Winqvist O, Puhakka L, Halonen M, Moro A, Kampe O, Eskelin P, Pelto-Huikko M, Peltonen L : Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. Hum Mol Genet 11 : 397-409, 2002
11. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins S, Turley SJ, von Boehmer H, Bronson R, Dierich A, Benoist C, Mathis D : Projection of an immunological self-shadow within the thymus by the Aire protein. Science 298 : 1395-1401, 2002
12. Kuroda N, Mitani T, Takeda N, Ishimaru N, Arakaki R, Hayashi Y, Bando Y, Izumi K, Takahashi T, Nomura T, Sakaguchi S, Ueno T, Takahama Y, Uchida D, Sun S, Kajiura F, Mouri Y, Han H, Matsushima A, Yamada G, Matsumoto M : Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. J Immunol 174 : 1862-1870, 2005
13. Derbinski J, Gabler J, Brors B, Tierling S, Jonnakuty S, Hergenhahn M, Peltonen L, Walter J, Kyewski B : Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. J Exp Med 202 : 33-45, 2005
14. Liston A, Lesage S, Wilson J, Peltonen L, Goodnow CC : Aire regulates negative selection of organ-specific T cells. Nat Immunol 4 : 350-354, 2003
15. Anderson MS, Venanzi ES, Chen Z, Berzins SP, Benoist C, Mathis D : The cellular mechanism of Aire control of T cell tolerance. Immunity 23 : 227-239, 2005
16. Niki S, Oshikawa K, Mouri Y, Hirota F, Matsushima A, Yano M, Han H, Bando Y, Izumi K, Matsumoto M, Nakayama K.I, Kuroda N, Matsumoto M : Alteration of intra-pancreatic target-organ specificity by abrogation of Aire in NOD mice. J Clin Invest 116 : 1292-1301, 2006
17. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS : Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435 : 220-223, 2005
18. Jiang W, Anderson MS, Bronson R, Mathis D, Benoist C : Modifier loci condition autoimmunity provoked by Aire deficiency. J Exp Med 202 : 805-815, 2005
19. Gallegos AM, Bevan MJ : Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J Exp Med 200 : 1039-1049, 2004
20. Kajiura F, Sun S, Nomura T, Izumi K, Ueno T, Bando Y, Kuroda N, Han H, Li Y, Matsushima A, Takahama Y, Sakaguchi S, Mitani T, Matsumoto M : NF-κB-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner. J Immunol 172 : 2067-2075, 2004
21. Yamada T, Mitani T, Yorita K, Uchida D, Matsushima A, Iwamasa K, Fujita S, Matsumoto M : Abnormal immune function of hemopoietic cells from alymphoplasia (*aly*) mice, a natural strain with mutant NF-κB-inducing kinase. J Immunol 165 : 804-812, 2000
22. Halonen M, Eskelin P, Myhre A.G, Perheentupa J, Husebye ES, Kampe O, Rorsman F, Peltonen L, Ulmanen I, Partanen J : AIRE mutations and

human leukocyte antigen genotypes as determinants of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy phenotype. J Clin Endocrinol Metab 87 : 2568-2574, 2002
23. Ramsey C, Hassler S, Marits P, Kampe O, Surh CD, Peltonen L, Winqvist O : Increased antigen presenting cell-mediated T cell activation in mice and patients without the autoimmune regulator. Eur J Immunol 36 : 305-317, 2006
24. Sakaguchi S : Naturally arising $CD4^+$ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 22 : 531-562, 2004
25. Mosmann TR, Coffman RL : TH1 and TH2 cells : different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7 : 145-173, 1989
26. von Boehmer H : T-cell lineage fate : instructed by receptor signals? Curr Biol 10 : R642-645, 2000
27. Hugo P, Kappler JW, McCormack JE, Marrack P : Fibroblasts can induce thymocyte positive selection *in vivo*. Proc Natl Acad Sci USA 90 : 10335-10339, 1993
28. Pawlowski T, Elliott JD, Loh DY, Staerz UD : Positive selection of T lymphocytes on fibroblasts. Nature 364 : 642-645, 1993
29. Vukmanovic S, Grandea AG III, Faas SJ, Knowles BB, Bevan MJ : Positive selection of T-lymphocytes induced by intrathymic injection of a thymic epithelial cell line. Nature 359 : 729-732, 1992
30. Takahama Y, Suzuki H, Katz KS, Grusby MJ, Singer A : Positive selection of $CD4^+$ T cells by TCR ligation without aggregation even in the absence of MHC. Nature 371 : 67-70, 1994
31. Zinkernagel RM, Althage A, Waterfield E, Kindred B, Welsh RM, Callahan G, Pincetl P : Restriction specificities, alloreactivity, and allotolerance expressed by T cells from nude mice reconstituted with H-2-compatible or -incompatible thymus grafts. J Exp Med 151 : 376-399, 1980
32. Martinic MM, Rulicke T, Althage A, Odermatt B, Hochli M, Lamarre A, Dumrese T, Speiser DE, Kyburz D, Hengartner H, Zinkernagel RM : Efficient T cell repertoire selection in tetraparental chimeric mice independent of thymic epithelial MHC. Proc Natl Acad Sci USA 100 : 1861-1866, 2003
33. Martinic MM, van den Broek MF, Rulicke T, Huber C, Odermatt B, Reith W, Horvath E, Zellweger R, Fink K, Recher M, Eschli B, Hengartner H, Zinkernagel RM : Functional $CD8^+$ but not $CD4^+$ T cell responses develop independent of thymic epithelial MHC. Proc Natl Acad Sci USA 103 : 14435-14440, 2006
34. Watanabe N, Hanabuchi S, Soumelis V, Yuan W, Ho S, de Waal Malefyt R, Liu YJ : Human thymic stromal lymphopoietin promotes dendritic cell-mediated $CD4^+$ T cell homeostatic expansion. Nat Immunol 5 : 426-434, 2004
35. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, Naji A, Caton AJ : Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. Nat Immunol 2 : 301-306, 2001
36. Apostolou I, Sarukhan A, Klein L, von Boehmer H : Origin of regulatory T cells with known specificity for antigen. Nat Immunol 3 : 756-763, 2002
37. Kishimoto H, Sprent J : A defect in central tolerance in NOD mice. Nat Immunol 2 : 1025-1031, 2001
38. Zucchelli S, Holler P, Yamagata T, Roy M, Benoist C, Mathis D : Defective central tolerance induction in NOD mice : genomics and genetics. Immunity 22 : 385-396, 2005
39. Liston A, Lesage S, Gray D.H, O'Reilly LA, Strasser A, Fahrer AM, Boyd RL, Wilson J, Baxter AG, Gallo EM, Crabtree GR, Peng K, Wilson SR, Goodnow CC : Generalized resistance to thymic deletion in the NOD mouse ; a polygenic trait characterized by defective induction of Bim. Immunity 21 : 817-830, 2004
40. Thomas-Vaslin V, Damotte D, Coltey M, Le Douarin NM, Coutinho A, Salaun J : Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. Proc Natl Acad Sci USA 94 : 4598-4603, 1997
41. Gillard GO, Farr AG : Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. J Immunol 176 : 5815-5824, 2006
42. Gillard GO, Farr AG : Contrasting models of promiscuous gene expression by thymic epithelium. J Exp Med 202 : 15-19, 2005