Aire-Dependent Alterations in Medullary Thymic Epithelium Indicate a Role for Aire in Thymic Epithelial Differentiation

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Aire-Dependent Alterations in Medullary Thymic Epithelium Indicate a Role for Aire in Thymic Epithelial Differentiation

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The prevalent view of thymic epithelial differentiation and Aire activity holds that Aire functions in terminally differentiated medullary thymic epithelial cells (MTECs) to derepress the expression of structural tissue-restricted Ags, including pancreatic endocrine hormones. An alternative view of these processes has proposed that Aire functions to regulate the differentiation of immature thymic epithelial cells, thereby affecting tissue-restricted Ag expression and negative selection. In this study, we demonstrate that Aire impacts several aspects of murine MTECs and provide support for this second model. Expression of transcription factors associated with developmental plasticity of progenitor cells, Nanog, Oct4, and Sox2, by MTECs was Aire dependent. Similarly, the transcription factors that regulate pancreatic development and the expression of pancreatic hormones are also expressed by wild-type MTECs in an Aire-dependent manner. The altered transcriptional profiles in Aire-deficient MTECs were accompanied by changes in the organization and composition of the medullary epithelial compartment, including a reduction in the medullary compartment defined by keratin (K) 14 expression, altered patterns of K5 and K8 expression, and more prominent epithelial cysts. These findings implicate Aire in the regulation of MTEC differentiation and the organization of the medullary thymic compartment and are compatible with a role for Aire in thymic epithelium differentiation. The Journal of Immunology, 2007, 178: 3007–3015.

A

lthough the importance of the thymic environment in governing the Ag specificity of the T cell repertoire and establishing self tolerance is well-established, the mechanistic bases for these processes remain poorly understood. The discovery that mutations in a single gene, the autoimmune regulator (AIRE), cause an autoimmune polyglandular syndrome in humans (1) led to the demonstration in mice that Aire activity is required for the expression of a diverse subset of tissue-restricted Ags (TRAs) by murine medullary thymic epithelial cells (MTECs), and that Aire contributes to the ability of cells within the thymic microenvironment to enforce negative selection (2).

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2 Address correspondence and reprint requests to Dr. Andrew G. Farr, Department of Biology Structure, Box 35-7420, University of Washington, Seattle, WA 98195-7420. E-mail address: farr@u.washington.edu
3 Abbreviations used in this paper: TRA, tissue-restricted Ag; MTEC, medullary thymic epithelial cell; TE, thymic epithelium; WT, wild type; CMF, Ca2+ - and Mg2+ -free; RQ, relative quantity; Gip, glucose-dependent insulinotropic peptide; LTR, lymphotxin receptor; Epcam, epithelial cell adhesion molecule; HPRT, hypoxanthine phosphoribosyltransferase; UEA, U. europaeus agglutinin.

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Aire DEFICIENCY ALTERS MEDULLARY TE

Table I. Contraction of the medullary epithelial compartment in the Aire−/− thymus

<table>
<thead>
<tr>
<th>Age at Analysis, in Weeks</th>
<th>Aire Genotype</th>
<th>Medulla-Cortex Ratio</th>
<th>Medulla as % of Whole Thymus</th>
<th>No. Sections Analyzed</th>
<th>Thymic Area Analyzed (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>+/+</td>
<td>0.191 ± 0.047</td>
<td>15.95 ± 3.15</td>
<td>28</td>
<td>119.5</td>
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<tr>
<td></td>
<td>−/−</td>
<td>0.125 ± 0.030</td>
<td>11.09 ± 2.33</td>
<td>30</td>
<td>161.1</td>
</tr>
<tr>
<td>8</td>
<td>+/+</td>
<td>0.315 ± 0.067</td>
<td>23.62 ± 4.20</td>
<td>19</td>
<td>84.3</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>0.161 ± 0.045</td>
<td>13.58 ± 3.47</td>
<td>23</td>
<td>120.6</td>
</tr>
<tr>
<td>10</td>
<td>+/+</td>
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<td>14.58 ± 2.89</td>
<td>21</td>
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<td>0.092 ± 0.030</td>
<td>8.36 ± 2.41</td>
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<tr>
<td>12</td>
<td>+/+</td>
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<td>14.64 ± 4.06</td>
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<td>53.7</td>
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<tr>
<td></td>
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<tr>
<td>16</td>
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<td>0.234 ± 0.049</td>
<td>18.89 ± 3.52</td>
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<td>57.3</td>
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<tr>
<td></td>
<td>−/−</td>
<td>0.094 ± 0.029</td>
<td>8.64 ± 2.15</td>
<td>21</td>
<td>62.8</td>
</tr>
</tbody>
</table>

* Sections were processed as described in Materials and Methods to quantify areas of 3G10 reactivity. Values for “Medulla-Cortex Ratio” and “Medulla as % of Whole Thymus” represent mean values ± SEM derived from the indicated number of sections. Differences between pooled Aire+/+ and Aire−/− samples (n = 6) or between age-matched samples were statistically significant (p < 0.05 and p < 0.001, respectively).

differentiated MTECs, Aire deficiency should not impact the overall organization or composition of the MTEC compartment, as proximal aspects of their differentiation program would be Aire independent. Furthermore, a direct role for Aire regulation of TRA expression in the thymus would obviate the requirement for the transcriptional regulation that normally controls their expression in corresponding extrathymic tissues. In contrast, the developmental model predicts that Aire deficiency would be associated with a more global perturbation of the medullary epithelial compartment because it would impact MTEC differentiation and/or survival. If the transcriptional hierarchies that regulate extrathymic epithelial differentiation/TRA expression are conserved by MTECs, the developmental model further predicts that MTECs would also express these transcriptional factors in addition to structural TRAs. Accordingly, the selective reduction of TRA expression by MTECs in Aire−/− mice would be accompanied by reduced expression of corresponding upstream regulatory transcription factors.

In this study, we have tested some of these corollary hypotheses regarding Aire function and TE differentiation. We report here that Nanog, Oct 4, and Sox2, transcription factors critical in maintaining the multipotentiality of progenitor cells and candidates for expression by epithelial progenitor cells in the thymus, are expressed by MTEC in a highly Aire-dependent manner. We also show that, in addition to pancreatic endocrine TRAs, such as insulin or glucagon, MTECs from normal thymi also express many of the regulatory transcription factors that are centrally involved in the development of the endocrine and exocrine compartments of the pancreas. Aire deficiency leads to dramatic reductions in the expression of most of these pancreatic regulatory transcription factors, suggesting that Aire may play a role in regulating MTEC expression of pancreatic TRAs in a more conventional manner, perhaps by acting as a proximal “master” control of more distal regulatory gene hierarchies in developing MTECs or by impacting the differentiation and/or survival of the cells that express these genes. In this study, we have also documented alterations in the organization and character of medullary epithelium in the Aire−/− thymus. These data establish that the impact of Aire deficiency extends beyond the lack of expression of a subset of TRAs in mature MTEC and effects changes in the thymic environment that would be compatible with a role for Aire in MTEC differentiation and/or survival.

Materials and Methods

Mice

Aire-deficient mice were generated as previously described (11) and maintained on a B6/129 background. An independently generated line of Aire-deficient mice (2) maintained on a C57BL/6 background was obtained from The Jackson Laboratory. Adult C57BL/6 mice were obtained from Charles River Laboratories or from our colony. All mice were maintained in the University of Washington specific pathogen-free facility and used in accordance with protocols approved by the University of Washington Institutional Animal Care and Use Committee.

Immunohistochemistry

Immunohistochemistry was performed on thymi from multiple littermates of both lines of Aire−/− mice and appropriate age-matched control (wild-type) mice as previously described. Reagents used in these analyses included G8.8 (available through the Developmental Studies Hybridoma Bank; www.uiowa.edu/~dshbwww/), 3G10 ERTR4, ERTR5, Trom1 (Developmental Studies Hybridoma Bank), polyclonal rabbit anti-KS (Covance), anti-MHC class II, anti-CD40, 10.11, biotinylated Ulex europeus (Vector Laboratories).

For immunofluorescence, the following secondary Abs were used: goat anti-rat IgG Alexa 488, goat anti-rat Alexa 546, goat anti-rabbit IgG Alexa 546, and streptavidin-Alexa 488 (all from Molecular Probes). For three-color analysis, goat Abs specific for rat IgM μ-chain or rat IgG γ-chains (Pierce) were conjugated with Alexa 647 or Alexa 488, respectively, according to the manufacturer’s protocol. For immunoperoxidase detection of 3G10, a three-step procedure was used, where unconjugated 3G10 Abs were detected by sequential application of goat anti-rat IgM μ-chain Abs (Pierce) modified by N-hydroxysuccinimide-digoxigenin (Boehringer Mannheim), followed by peroxidase-conjugated sheep anti-digoxigenin F(ab′)2 Ab (Boehringer Mannheim) and color development with 3,3′diaminobenzidine (Sigma-Aldrich).

Morphometry

Thymic sections were collected at 70-μm intervals through both lobes of age-matched Aire+/+ and Aire−/− thymi. After processing to demonstrate 3G10 staining as previously described (12), the sections were photographed at ×25 magnification and images were printed at the same magnification. Thymic lobe profiles were cut out and weighted, and then the medullary compartment identified by 3G10 staining was cut out and the resulting pieces were also weighed. The weight of the paper was calibrated to image area by weighing squares of paper that corresponded to areas defined by a stage micrometer. Numbers of sections analyzed and the area that these sections encompassed are listed in Table I. Statistical significance was determined by the Wilcoxon rank-sum test.

Sorting and flow cytometry

Enzymatic dissociation of thymi was performed as described previously (8). Briefly, thymi from 4- to 10-wk, age- and gender-matched mice were diced in HBSS (Ca²⁺ and Mg²⁺-free (CMF) plus 2% FBS plus 10 mM...
HEPES buffer, washed by gravity sedimentation, and then digested with collagenase D (Roche) in CMF HBSS, followed by a mixture of collagenase and neutral dispase (Roche) to obtain a single-cell suspension. Single-cell suspensions were treated with rat anti-FcR II/III mAb (clone 24G2) before Ab labeling to reduce nonspecific Ab binding. After staining, cells were washed twice in HBSS CMF and suspended in the same containing 7-aminoactinomycin D (Molecular Probes), at a final concentration of 2 μg/ml.

**Isolation of RNA**

Techniques for RNA isolation, amplification, and PCR analyses have been described previously (8). Primer sequences are available upon request. Determination of relative quantity (RQ) values has been described previously (13) and SDs of real time data were calculated according to the manufacturer’s instruction (www.appliedbiosystems.com).

**Results**

**Aire-deficient MTECs express reduced levels of transcription factors that confer multipotentiality**

The presence of a progenitor MTEC population in the adult thymus has been inferred by demonstrations of cells with progenitor activity in the fetal thymus (14–16) and recently demonstrated in adult thymus (17), although the full developmental potential of these cells has not been determined. We have previously established that the Nanog, Oct4, and Sox2 core transcriptional factors

**FIGURE 1.** Transcription factors associated with multipotentiality are expressed by MTEC in an Aire-dependent manner. A, Flow cytometric separation of enzymatically dissociated G8.8+ thymic stromal cells from Aire+/+ and Aire−/− thymi. Profiles have been gated on CD45+ cells. Although the representation of G8.8+CD45+ epithelial cells from these two sources appear roughly equivalent, variability between experiments and the low sensitivity of directly conjugated anti-cortical reagent Abs do not allow conclusions to be drawn regarding the relative representation of TE subsets. B, Semiquantitative RT-PCR analyses of sorted Aire+/+ and Aire−/− MTEC. Sample dilutions: undiluted, 1/5 and 1/25. C, Quantitative real-time PCR analysis of sorted Aire+/+ and Aire−/− MTEC. Expression levels were normalized to Epcam and are represented as the percentage of WT expression. Data represent three independent samples. SD for any sample was <2% from the average value.

**FIGURE 2.** Aire-deficient MTEC fail to express multiple pancreatic endocrine hormone genes. A, RT-PCR characterization of MTEC population used for this analysis. The flow cytometric characterization of these cells has been described (8). Consistent with their medullary character, these cells express K5, MHC class II, and Aire. Aire signal was undetectable in samples from Aire−/− mice. Sample dilutions: undiluted, 1/5 and 1/25. B, Semiquantitative RT-PCR analysis of pancreatic endocrine gene products expressed by Aire+/+ and Aire−/− MTEC. * Gcg, Glucagon; Ins2, insulin 2; Ppy, pancreatic polypeptide; SST, somatostatin. Samples were normalized to HPRT (see A) or Epcam, which gave equivalent results (data not shown). Sample dilutions: undiluted, 1/5 and 1/25. C, Quantitative real-time PCR analyses of pancreatic endocrine gene produces expressed by Aire+/+ and Aire−/− MTEC. Samples labeled as in B. Data represent three independent samples. SD for any sample was <2% from the average value.
of multipotentiality (18, 19), are expressed within the MTEC population and have proposed this to reflect the presence of a developmentally flexible progenitor epithelial population in the adult thymus (8). To explore the hypothesis that Aire could influence TE differentiation, we determined the impact of Aire deficiency on the expression of these transcription factors by MTECs. Medullary epithelial cells were isolated from enzymatically dissociated thymus by flow cytometry. A representative set of Aire+/+ and Aire−/− thymic samples is shown in Fig. 1A. Although this approach allowed recovery of a defined subset of MTEC, variability of cell recovery and variable efficiency of MTEC recovery do not allow conclusions regarding relative representation of TE subsets based on this approach with our current methodologies. That being said, we did not observe any reproducible differences between Aire+/+ and Aire−/− thymi prepared in this manner. Fig. 1, B and C, depicts results of semiquantitative and real-time PCR of MTEC cDNAs from enzymatically dissociated Aire+/+ and Aire−/− thymi. These results confirmed the expression of Nanog, Oct4, and Sox2 by Aire+/+ MTECs (8) and revealed a dramatic reduction in their expression within the Aire−/− MTEC population. We performed similar analyses of the expression of other transcription factors previously implicated in thymic organogenesis (FoxN1, Pdx1, Pax9, and lymphotoxin-β receptor (LTβR); reviewed in Refs. 20 and 21) or in the differentiation of multiple epithelial lineages (Foxa1 and Foxa2; referenced in Ref. 8) and determined that Aire+/+ and Aire−/− MTECs demonstrated comparable expression levels of these transcription factors (Fig. 1D). Based on results of the dilution series analyses, real-time PCR analyses were not performed for these latter groups of molecules.

Aire deficiency leads to severe reductions in the pancreatic transcriptional pathway

The properties of the MTEC populations analyzed here have been described previously (8) and are also shown in Fig. 2A. Confirming previous reports (2, 4), real-time quantitative PCR analyses of cDNA derived from sorted Aire+/+ and Aire−/− MTEC showed that that MTEC express a number of genes characteristically associated with the endocrine portion of the pancreas in an Aire-dependent manner (Fig. 2, B and C). The endocrine pancreatic compartment was represented by glucagon, pancreatic polypeptide, somatostatin, and glucose-dependent insulinothropic peptide (Gip). Gip is Aire dependent and is regulated in the intestine/gastrointestinal cells by Pdx1, a transcription factor required for pancreatic development. Normalizing levels of pancreatic gene expression to levels of hypoxanthine phosphoribosyltransferase (HPRT) or Epcam (a reliable marker of MTEC), we found that Aire-deficient MTEC expressed virtually no pancreatic polypeptide, somatostatin, or Gip; the levels of glucagon and insulin2 expressed by MTEC were reduced to ~40 and 10% of WT levels, respectively.

If the general mechanism that regulates expression of pancreatic TRAs by pancreatic epithelial cells is conserved in MTEC, the regulatory hierarchy of transcription factors that control pancreatic endocrine development should also be expressed by MTEC. Some of the transcription factors that are critical for pancreatic development are shown in Fig. 3A and are described below. Pdx1, along with Ptf1a, is required for pancreatic organogenesis; Pdx1 also has a critical role in later stages of endocrine cell development, particularly for the development and function of mature β or δ cells in the islets (22–24). Expression of Ptf1a is also required at later stages in the differentiation of exocrine lineages (25). Neurogenin 3 is a transcription factor that is required for the development of all four endocrine lineages in pancreas (26), while NeuroD1 expression is required for the establishment of mature endocrine cells, particularly β cells (27). The main phenotype in Nkx2.2- and Nkx6.1-deficient mice is a severe decrease in the production of insulin, which has been attributed to impaired maturation of β cells (28, 29). The paired-box transcription factors, Pax4 and Pax6, also play important roles in the specification of the four endocrine lineages. Pax6 expression by endocrine progenitor cells is important for the formation of all endocrine lineages, particularly α cells (30), while Pax4 is required for the formation of β and δ cells (31).

We analyzed cDNAs generated from bulk populations of Epcam+/CD45− MTECs sorted from groups of age-matched adult Aire+/+ mice for the expression of a subset of these pancreatic developmental transcription factors. As shown in Fig. 3B,
we found that normal MTEC expressed many of the transcription factors that are involved in the specification and/or differentiation of endocrine and exocrine pancreatic lineages, including Pdx1, Nkx2.2, Nkx6.1, Pax4, and Pax6. A similar analysis of sorted MTEC from Aire<sup>−/−</sup> mice revealed that expression of most of these pancreatic transcription factors was dramatically reduced in Aire-deficient MTEC, with the exceptions of Ptf1a and NeuroD1, which displayed ∼2-fold differences. These semiquantitative RT-PCR results were confirmed by real-time PCR analyses (Fig. 2C), where expression of Pdx1, Nkx2.2, Nkx6.1, Pax4, and Pax6 by Aire<sup>−/−</sup> MTEC was reduced to ∼5% of WT levels. It is noteworthy that severe reductions in Pdx1 expression by the Aire-deficient MTEC population was mirrored by severe reductions in the expression of three endocrine structural genes, Ins, Sst, and Gip, that are directly regulated by Pdx1 and whose promoters are binding targets for Pdx1 (32–34). These results demonstrate that the genes affected by Aire deficiency in MTEC extend beyond structural products of peripheral lineages to include the regulatory elements that control expression of these genes in peripheral tissues.

The organization and composition of the medullary thymic epithelial compartment is impacted by Aire deficiency

Previous analyses of the Aire<sup>−/−</sup> thymus concluded that the organization was unremarkable, based on distinct cortical and medullary compartments in histologic samples (2, 11, 35). Here, we have used immunohistochemistry to analyze the organization and composition of the Aire-deficient thymic stromal compartment in more detail.

In the Aire<sup>+/+</sup> thymus, high levels of Epcam expression delineated the medullary epithelial compartment (Fig. 4, A and E). Within this medullary compartment, additional heterogeneity in the Aire<sup>+/+</sup> thymus was demonstrated by reactivity with the fusose-specific U. europeus agglutinin (UEA) (36) (Fig. 4, B and E) or with the mAb 10.1.1, which reacts with a subset of MTEC (37) (Fig. 4G). The staining patterns observed with UEA (Fig. 4B) and 10.1.1 (Fig. 4H) in the Aire<sup>−/−</sup> thymus indicated that the representation of MTEC heterogeneity was fundamentally altered. Although the representation of MTEC that expressed low levels of UEA staining were comparable in Aire<sup>+/+</sup> and −/− mice, the clusters of MTEC strongly labeled with this lectin was not detected in the Aire<sup>−/−</sup> thymus (compare Fig. 4, C and D). Similarly, the Aire<sup>−/−</sup> thymus displayed a marked reduction in the frequency of 10.1.1<sup>+</sup> cells (compare Fig. 4, G and H).

The mAb 3G10, which reacts with medullary TE and has been provisionally identified as an anti-K14 mAb (12) defines the major population of MTEC in both WT and Aire<sup>−/−</sup> thymus. The prominence of epithelial cysts and the less dense medullary compartment in the Aire<sup>−/−</sup> thymus is evident in thymic sections labeled
with 3G10 (compare Fig. 5, A and B with C and D). Morphometric analyses of multiple sections of Aire+/− and −/− thymic tissue that were processed to demonstrate 3G10 with peroxidase-labeled Abs revealed a marked alteration of the medullary compartment of Aire−/− thymus. As shown in Table I, Aire−/− thymi had a smaller 3G10 medullary compartment, defined either as a cortical/medullary ratio or as the percent contribution of the medullary area to total thymic area. The reduction of this MTEC compartment in the Aire−/− thymus was apparent in the six pairs of Aire+/− and −/− thymi examined in this manner and was statistically significant in all instances (p < 0.05 between Aire+/− and −/− groups and p < 0.001 between each age-matched pair). These alterations were evident in mice younger than 12 wk of age (when autoimmune activity is first detected in Aire−/− mice; Ref. 2), and as early as 1 wk of age (data not shown), so it is unlikely that the alterations of the medullary compartment are secondary to the onset of autoimmune phenomena. Real-time PCR analyses of K8 and K14 expression in two independent sorted populations of EpcamCD45−/− MTEC from Aire−/− and +/+ thymi confirmed the trends in keratin expression observed immunohistochemically. Matched cDNA samples were first normalized to the expression of Epcam and then RQ values were obtained for K8 and K14. In both sets of sorted MTEC, the Aire−/− cells displayed a smaller RQ value for K14, indicating a fundamental alteration in the character of the Aire−/− MTEC (Table II).

To further assess the impact of Aire deficiency on thymic epithelial heterogeneity, we examined the expression of K5 and K8 by thymic epithelial cells. It had been previously reported that K8 expression is largely restricted to cortical epithelial cells, with a

<table>
<thead>
<tr>
<th>Keratin</th>
<th>Set</th>
<th>Aire+/−</th>
<th>Aire−/−</th>
</tr>
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<tr>
<td>8</td>
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<td>0.61</td>
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</table>

* Sorted CD45−/−CDR1−/−Epcam+ TE cells from Aire+/− and −/− mice were processed to generate cDNA and then analyzed with real-time RT-PCR to determine levels of K8 and K14 message expression. RQ values were determined, setting the cycle threshold of the Aire+/− sample to 1.
small subset of K8+ cells within the medulla. In contrast, K5 expression was found to be largely restricted to the medulla with a small subset of K5+K8+ cells at the corticomedullary boundary and scattered in the cortex. Based on the expression patterns of these cytokeratins under different experimental conditions, it has been proposed that the K5+K8+ thymic epithelial cells represent a progenitor population that give rise to more differentiated thymic epithelial cells that expressed either K5 or K8 (38–40). We confirmed the widespread expression of K5 throughout the medulla and cells coexpressing K5 and K8 at the corticomedullary junction or scattered in the cortex (Fig. 6, A–F). We also confirmed the universal expression of K8 by cortical cells and a small subset of medullary TE that possessed a K5+K8+ phenotype with a globular profile (Fig. 6, A–F). In contrast to previous reports, we found variable but widespread expression of K8 by medullary TE (Fig. 6, A and D). Many of the more stellate medullary TE cells that expressed high levels of K5 variably expressed K8 as well (compare Fig. 6, A and D with B and E).

The pattern of K5/K8 expression by MTEC in the Aire−/− thymus highlighted a number of unique features of the medullary epithelial compartment. In contrast to the stellate medullary epithelial cells in the Aire−/+ thymus that were joined by fairly broad cellular processes and that formed a fairly dense and uniform network (Fig. 6, C and F), Aire−/− MTEC were often quite spindly and were joined to each other by thin and sometimes long processes, leaving larger areas of medulla devoid of cytokeratin+ epithelial cells (Fig. 6, I and L). Furthermore, the frequency of MTEC that were globular, “rounded-up” without any visible cellular projections, and expressing high levels of K8 were more prominent in the medullary compartment of Aire−/− thymi (compare Fig. 6, D and J).

Two populations of globular K5−K8− MTEC defined by their reactivity with UEA are present within the normal thymic medulla (Ref. 38 and Fig. 7, A–D), where K5−K8−UEA− MTEC and K5−K8−UEA− MTEC are indicated by arrows and arrowheads, respectively. Both of these populations of MTEC were also present in the Aire−/− thymus (Fig. 7, E–H). The globular MTEC expressed K8 and low levels of K5 that were found in the Aire−/− thymus are indicated by double-headed arrows in Fig. 7, E–H. Occasionally, this population of MTEC also displayed reactivity with UEA. The demonstration here of K8−K5−UEA− and K8−K5−UEA− subsets of globular MTEC represents a greater degree of heterogeneity than that originally described for this population (38).

Discussion

There has been a long-standing assumption, arising from the relative radiation resistance of TE, that the epithelium in the adult thymus is a largely postmitotic population with little turnover. Accordingly, the derepression model of promiscuous gene expression has been reasonably proposed as a logical mechanism to account for TRA expression by a static population of MTEC. However, recent studies have demonstrated continued MTEC turnover in the adult thymus (6, 8, 9), indicating that the medullary compartment represents a steady state of MTEC at different stages of differentiation and implies the presence of a resident epithelial progenitor population in the adult thymus. Importantly, it was demonstrated that the MHC class II+ population of MTEC, which have been proposed to represent mature MTEC expressing the highest levels of TRAs and Aire (4), also display a high level of proliferation and have been proposed to represent a transit-amplifying subset of MTEC (6). The representation of this MTEC subset in several mouse “knockout” models also suggest that the MHC+ population of MTEC, which have been proposed to represent mature MTEC expressing the highest levels of TRAs and Aire (4), also display a high level of proliferation and have been proposed to represent a transit-amplifying subset of MTEC (6). The representation of this MTEC subset in several mouse “knockout” models also suggest that the MHC+ population of MTEC, which have been proposed to represent mature MTEC expressing the highest levels of TRAs and Aire (4), also display a high level of proliferation and have been proposed to represent a transit-amplifying subset of MTEC (6). 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This possibility would be congruent with previous evidence for coordinated expression of lineage-related TRAs, where single-cell RT-PCR analyses of isolated MTEC revealed coordinated expression of developmentally related, Pdx1-dependent endocrine Ags that did not possess chromosomal proximity (8). Furthermore, the Aire dependence of transcription factors crucial for endocrine pancreatic differentiation/function in MTECs suggests that the reduction of pancreatic TRA expression

![Figure 7](http://www.jimmunol.org/)

**Figure 7.** Demonstration of K5 (red), K8 (green), and UEA (blue) in Aire+/+ (A–D) and Aire−/− (E–H) thymus. Globular K5+, K8+, UEA+ MTEC are indicated by arrows. Globular K5+, K8+, UEA+ MTEC are indicated by arrowheads. Globular K5+, K8+, MTEC found in the Aire−/− thymus are indicated by double-headed arrows. This last population typically displays low levels of UEA binding.
by Aire$^{-/-}$ MTECs may be secondary to disruption of this transcriptional hierarchy. Additional single-cell transcriptional analyses, along with assessment of the spectrum of pancreatic TRAs expressed by MTEC from mice lacking these tissue-restricted regulatory transcription factors, should clarify this issue and are in progress.

The proposition that Aire plays a central role in MTEC differentiation is based in part on the contraction of the medullary compartment and reduced MTEC heterogeneity in the Aire$^{-/-}$ thymus. This phenotype of the Aire$^{-/-}$ thymus suggests that Aire influences the dynamic process of MTEC differentiation and thereby affects the composition of MTEC. Although many aspects of thymic epithelial differentiation are poorly understood, it is clear that the NF-kB-signaling pathway plays a central role. The profound reduction of the MTEC compartment in the RelB$^{-/-}$ thymus encompasses a dramatic loss of Aire expression and a paucity of UEA$^{+}$MTEC (41, 42), while deficiencies of signaling pathways that regulate RelB expression variably recapitulate the RelB$^{-/-}$ thymic phenotype. Thymi from mice deficient in TNFR-associated factor 6 (43), NF-kB-inducing kinase (44), or IkB kinase alpha (45) closely resemble the RelB$^{-/-}$ thymus, while the thymic phenotype of Ltarda/Ltbr-deficient mice is less severe (46, 47) and CD40$^{-/-}$ thymus appear to be normal (referenced in Ref. 44). The Aire-deficient thymic phenotype is certainly milder that that observed in the RelB$^{-/-}$, TNFR-associated factor 6$^{-/-}$, NF-kB-inducing kinase$^{-/-}$, or IkB kinase alpha$^{-/-}$ mice and bears some similarity to the thymic phenotype of mice lacking Ltarda or Ltbr.

The looser organization of the contracted medullary compartment of the Aire$^{-/-}$ thymus may account for more efficient enzymatic dissociation Aire$^{-/-}$ thymic tissue that we have observed (G. O. Gillard and A. G. Farr, unpublished observations). It is worth noting that these alterations of the thymic stromal compartment described here are not accompanied by significant perturbations in the representation of thymocytes subsets defined by flow cytometric analyses (11).

The contracted medullary compartment of the Aire$^{-/-}$ thymus lacked the confluent stellate MTEC seen in the normal thymus and had increased representation of globular “rounded-up” MTEC expressing high levels of K8. The variable and widespread coexpression of K5 and K8 within the medullary compartment that we observed in both the Aire$^{+/+}$ and Aire$^{-/-}$ thymus is difficult to reconcile with the view that K5$^{+}$K8$^{+}$ MTEC represent a precursor population and that K8 expression is largely restricted to cortical TE (38–40). We feel this discrepancy reflects the sensitivity of detection because the same primary Abs were used in both studies. The Alexa fluorochromes used in this study provide stronger fluorescent signals than the FITC or Texas Red dyes used in the previous report. We have observed a similar pattern of K8 expression by medullary TE with a three-step immunoperoxidase method (unconjugated primary Ab, digoxigenin-conjugated antirat IgG Abs, and a peroxidase-conjugated anti-digoxigenin F(ab')2 Ab; data not shown). Thus, while it is clear that the distribution and morphology of MTEC expressing these two keratins are perturbed in the Aire$^{-/-}$ thymus and likely reflect an altered differentiation program, we feel the developmental relationship between the TE populations defined by K5 and K8 expression is not clear.

The increased prevalence of cystic epithelial structures in the Aire$^{-/-}$ thymus is another indication that MTEC differentiation has been affected. This view is based on our previous observation that this small epithelial compartment in the normal thymus (10) resembles the predominant epithelial compartment in the Foxn1$^{-/-}$ thymus. The profound proximal defect in epithelial differentiation in the Foxn1$^{-/-}$ thymus leads to accumulation of “thymic” epithelium with phenotypic properties of respiratory epithelium (48), perhaps reflecting an alternate fate choice by third pharyngeal pouch endoderm incapable of expressing functional Foxn1.

The alterations in MTEC composition/organization observed in the Aire$^{-/-}$ thymus compared with the RelB$^{-/-}$ thymus indicate that the general program of MTEC differentiation is more subtly impacted by Aire deficiency. From this perspective, the Aire dependence of nanog, Oct4, and Sox2 expression by MTEC is intriguing. If the activity of these transcription factors in MTEC recapitulates their role in maintaining the pluripotentiality of stem cells, their lack of expression by Aire$^{-/-}$ MTEC may alter the extent of MTEC heterogeneity or the range of TRAs they express, perhaps by reducing the spectrum of developmental programs that developing MTEC can undertake or by changing the kinetics of differentiation processes. One possibility is that Aire impacts TRA expression by influencing the tempo or efficiency of MTEC differentiation. If the expression of some TRAs occurs transiently during proximal stages of MTEC differentiation, an accelerated tempo of MTEC differentiation due to Aire deficiency would reduce the permissive period for TRA expression and reduce the frequency of MTECs expressing Aire-dependent TRAs at a given time. Precocious or accelerated MTEC differentiation could also result in a reduced burst size of differentiating MTECs and thus account for the contracted 3G10$^{+}$ MTEC population observed in the Aire-deficient thymus. It is likely that this reduced MTEC compartment would be less efficient in supporting negative selection in general, and negative selection of TRA-reactive thymocytes in particular, due to the relatively rare expression of any individual TRA (3, 8, 49). In this regard, the contraction of medullary epithelium in the Aire-deficient thymus could provide a mechanistic explanation for the impaired negative selection of OVA-specific transgenic T cells in Aire-deficient mice that is independent of OVA transcription levels (5).

The alterations in thymic medullary epithelial composition and organization seen in the Aire$^{-/-}$ thymus clearly indicate that the activity of Aire in MTECs extends beyond its previously ascribed role as a regulator of TRA expression in mature MTECs. These alterations in epithelial composition and organization are not accounted for by derepression models as presently constituted, but are consistent with a developmental model for Aire function. Although the transcriptional data presented here can be interpreted to reflect a higher order of transcriptional derepression effected by Aire, and thus cannot exclude a role for Aire as a “randomizer” of gene expression, they provide circumstantial evidence that is consistent with an alternative model of Aire function that can be experimentally tested. The novel properties of MTECs and the thymic consequences of Aire deficiency that are documented here need to be accounted for when developing and refining models for TE differentiation and Aire function in the thymus.

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