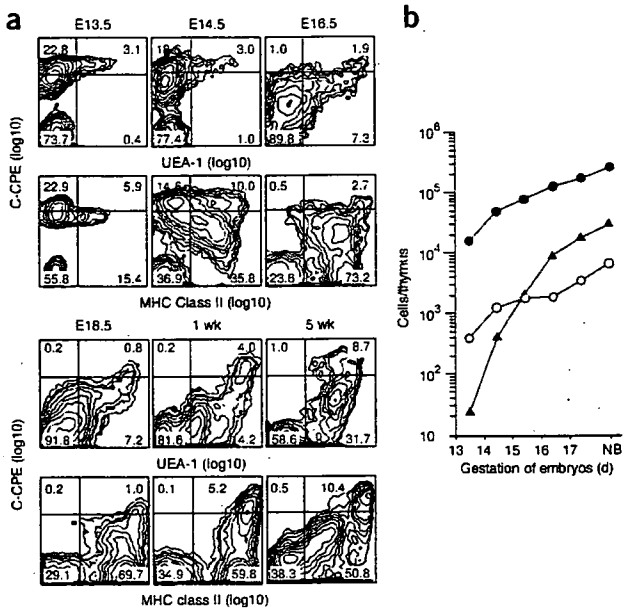

Figure 4 C-CPE^{hi} UEA-1$^+$ TECs develop earlier but proliferate more slowly during embryonic ontogeny than do C-CPE^{lo} UEA-1$^+$ TECs. (**a**) Flow cytometry of mouse thymic stromal cells at various developmental stages stained with anti-CD45, biotinylated C-CPE and FITC–UEA-1 or anti–MHC class II. Data represent profiles of C-CPE versus UEA-1 or C-CPE versus MHC class II in the CD45$^-$ gate. Numbers in quadrants indicate percent cells in each. Data are representative of three experiments. (**b**) Numbers of C-CPE^{hi} UEA-1$^+$ TECs (open circles) and C-CPE^{lo-neg} UEA-1$^+$TECs (filled triangles) per thymus, calculated from total CD45$^-$ cell numbers (filled circles) and flow cytometry. NB, newborn. Each symbol represents the mean of three independent experiments with pooled thymic lobes from a litter of embryos or newborns.

(BrdU), we did not detect BrdU incorporation in Cld3,4$^+$ Aire$^+$ mTECs (**Fig. 2d**). However, there was sporadic incorporation in Cld3,4$^+$ Aire$^-$ mTECs, suggesting that the small Cld3,4$^+$ Aire$^-$ mTEC population might include transitional cells still in cycle.

Tight-junction component expression during thymic ontogeny

Next we investigated the expression of tight-junction components during thymic ontogeny. Cld3,4 proteins were expressed first at tight junctions of cell-adhesion sites of the pharyngeal endoderm (embryonic day 9.5 (E9.5); data not shown). At E10.5, high expression of Cld3,4 and occludin was confined to endoderm cells at an apical layer of the bilayered thymic rudiment outgrowing from the third pharyngeal pouch endoderm (**Fig. 3a**). Cld3,4 and occludin were concentrated at the most apical part of the cell-adhesion sites, which is characteristic of tight junctions (**Fig. 3a**). Cytokeratin 8 and E-cadherin, in contrast, were expressed uniformly in all endoderm cells. As the anlage became progressively stratified and isolated at E11.5, the most apical epithelial cells positioned at the innermost layer continued to express Cld3,4 and occludin (**Fig. 3b**). At E12.5, the overall architecture of the anlage was substantially reorganized into a cluster structure. Accordingly, the Cld3,4$^+$ epithelial layer was 'crushed' into a cluster at the central region of the anlage; most cells in this region concomitantly expressed MTS10 (**Fig. 3c**), representing the first TECs to express a known mTEC marker in ontogeny. At E13.5, the Cld3,4$^+$ TECs were scattered throughout the anlage in small clusters (**Fig. 3d**). At later fetal stages, the Cld3,4$^+$ MTS10$^+$ cells seemed to be surrounded by increasing numbers of Cld3,4^{lo-neg} MTS10$^+$ TECs (**Fig. 3e**), and at the newborn stage, most if not all of the Cld3,4$^+$ cells scattered in the coalesced medulla were MTS10$^-$ (**Fig. 3f**).

Flow cytometry indicated that C-CPE^{hi} TECs were the first cells to express UEA-1 ligand, another marker of mTECs, at E13.5, and that C-CPE^{hi} UEA-1$^+$ TECs had high expression of MHC class II by E16.5 to E18.5 (**Fig. 4a**). Although the generation of C-CPE^{hi} UEA-1$^+$ TECs preceded that of C-CPE^{lo}UEA-1$^+$ TECs, the proliferation rate of the former transiently slowed down at E14.5–E16.5, when Aire expression was expected to begin (**Fig. 4b**). C-CPE^{lo} UEA-1$^+$ TECs, in contrast, demonstrated exponential proliferation throughout the fetal stages, eventually becoming a main mTEC population (**Fig. 4b**), consistent with the immunostaining data.

E13.5 Cld3,4$^+$ TECs give rise to Aire$^+$ mTECs

To directly investigate the differentiation potential of fetal Cld3,4$^+$ TECs, we isolated the following three fractions of TECs from E13.5 C57BL/6 (B6; H-2K^b) embryos by cell sorting: R1 (C-CPE^{hi}UEA-1$^+$), R2 (C-CPE^{hi}UEA-1$^-$) and R3 (C-CPE^{lo}UEA-1$^-$; **Fig. 5a**). Reanalysis of the sorted cells by immunostaining showed that all cells in the R1 and R2 fractions expressed Cld3,4, as expected, whereas only 8% of the R3 fraction expressed Cld3,4. Also, as much as 96% of the cells in R1 fraction expressed MTS10, whereas only 3% of the cells in R2 and none in R3 fractions expressed MTS10 (**Supplementary Fig. 3** online). We reaggregated sorted cells (1×10^4) of each fraction with total enzyme-digested cells (3×10^5) of E13.5 BALB/c (H-2K^d) thymic lobes and grafted them under the kidney capsules of nude mice. Then, 3 weeks later, we assessed the fates of donor cells in the ectopic thymus using antibody to H-2K^b (anti-H-2K^b). We confirmed that the H-2K^b-specific antibody was capable of specifically detecting both cTECs and mTECs derived from B6 donors (**Supplementary Fig. 4** online). H-2K^b TECs derived from R1 and R2 donors were present exclusively in the medulla, suggesting that the C-CPE^{hi} TECs at E13.5 were committed to the mTEC lineage (**Fig. 5b**). In contrast, donor cells from the R3 fraction were distributed throughout the medulla and cortex, indicating that common TEC progenitors may be present in the C-CPE^{lo} fraction. TECs derived from the R2 and R3 fractions consistently formed much larger clusters than did those from R1, indicating a greater proliferative capacity. Most mTECs derived from the R1 fraction had a Cld3,4$^+$MTS10$^-$ surface phenotype (**Fig. 5c**, top), and three-color analysis showed that many of them expressed Aire (**Fig. 5d**). In contrast, R2-derived mTEC clusters contained Cld3,4$^-$MTS10$^+$ mTECs in addition to Cld3,4$^+$ mTECs (**Fig. 5c**, bottom). These results suggested that Cld3,4$^+$ TECs in the R2 fraction contained the progenitors of Cld3,4$^+$ and Cld3,4$^-$ mTECs, or the common progenitors of both mTECs. Cld3,4$^+$ mTECs, including Aire$^+$ cells, were also present at variable frequencies in the clusters derived from R3 donors (**Supplementary Fig. 5** online). Our results are consistent with the idea of common TEC progenitor activity in R3 fraction, although that remains to be proven by a clonal analysis.

To confirm the precursor-progeny relationship between E13.5 Cld3,4$^+$UEA-1$^+$MTS10$^+$ cells and Cld3,4$^+$MTS10$^-$Aire$^+$ mTECs, we fed recipient mice BrdU for the first 9 d after thymic lobe grafting. Many of the H-2K^{b+}Cld3,4$^+$Aire$^+$ mTECs incorporated BrdU, indicating that they developed with proliferation *in vivo* (**Fig. 6**). Because *Aire* expression was not yet detected at E13.5, and Cld3,4$^+$Aire$^+$ mTECs were essentially nondividing (**Fig. 2d**), it was very unlikely that the BrdU$^+$ cells represented mere carryover of the very few Aire$^+$

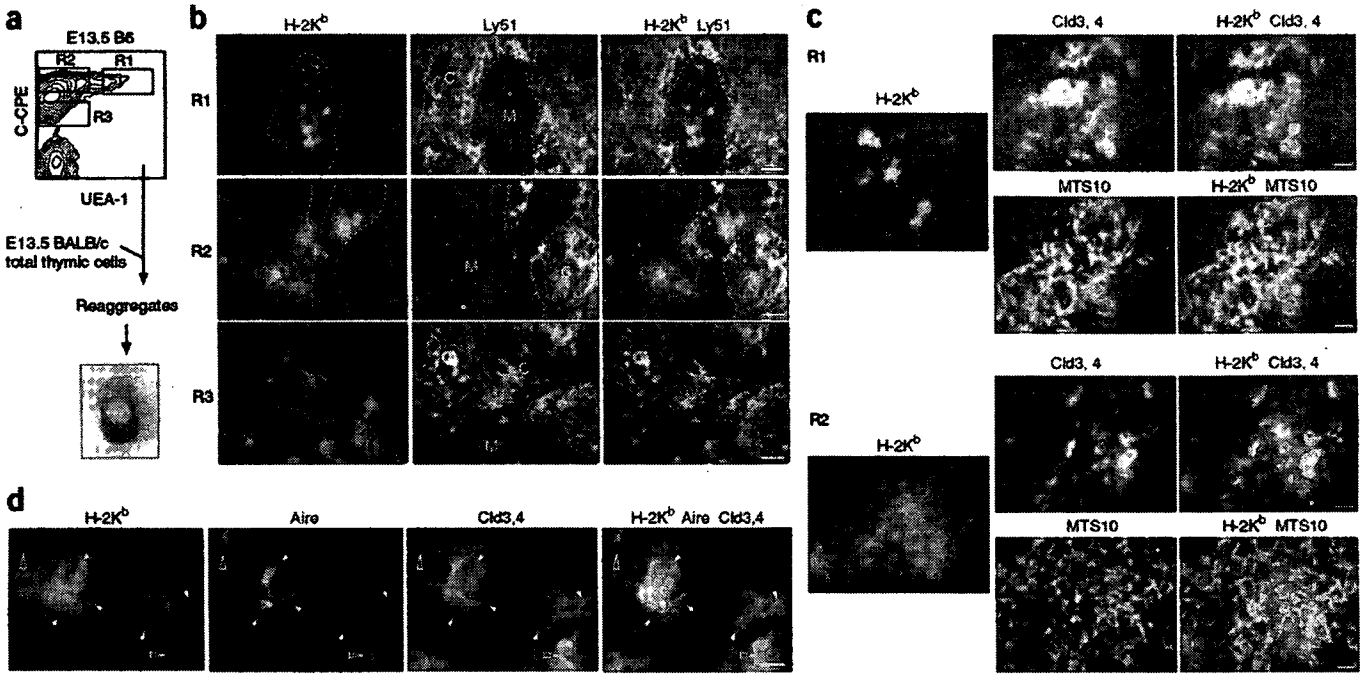

Figure 5 C-CPE^{hi} UEA-1$^+$ TECs sorted at E13.5 'preferentially' generate Cld3,4$^+$ MTS10$^-$ mTECs expressing Aire. (**a**) Generation of ectopically developed thymi. Thymic stromal cells from E13.5 B6 mouse embryos were stained with biotinylated C-CPE and FITC–UEA-1 and fractions (gates R1–R3) were sorted; 1×10^4 cells from each fraction were reaggregated together in culture for 24 h with 3×10^5 total thymic cells from E13.5 BALB/c embryos and then were implanted under the kidney capsules of BALB/c nude mice; 3 weeks later, the ectopically developed thymi were analyzed. (**b**) Immunostaining of the reconstituted thymi containing donor TECs from each fraction in **a** with anti-H-2K^b and anti-Ly51 (cTEC marker). Dotted lines indicate cortico-medullary borders. (**c**) Three color-immunostaining (with anti-H-2K^b, anti-Cld3,4 and anti-MTS10) of reconstituted thymi from aggregates containing R1 (top) or R2 (bottom) donor TECs. All images show the same field of a section; anti-Cld3,4 and anti-MTS10 are the same color to facilitate comparison. (**d**) Three color-immunostaining (with anti-H-2K^b, anti-Cld3,4 and anti-Aire) of aggregates containing R1 donor TECs. Filled and open arrowheads indicate B6 (donor)–derived and BALB/c (recipient)–derived Cld3,4$^+$Aire$^+$ mTECs, respectively. Scale bars, 50 μm (**b**), 20 μm (**c**) and 10 μm (**d**). Reconstitution experiments were done three times for each fraction with essentially similar results.

TECs that might be present in the R1 fraction. These results suggested that Cld3,4$^+$UEA-1$^+$MTS10$^+$ TECs at E13.5 represented the immediate mTEC progenitors specifically committed to a unique Cld3,4$^+$MTS10$^-$ mTEC lineage expressing Aire.

Signaling requirements of Cld3,4$^+$ MTS10$^-$ mTEC development

We finally investigated the development of Cld3,4$^+$ TECs in 'aly/aly' mice, which lack functional NF-κB-inducing kinase, and *Traf6*$^{-/-}$ mice, both of which are known to have defective Aire expression[22,23]. At E13.5, the numbers and distribution profiles of Cld3,4$^+$ TECs in the mutant mice of both lines were indistinguishable from those of their heterozygous littermate controls (**Fig. 7a**). By E16.5, however, Cld3,4$^+$ TECs were considerably fewer in aly/aly mice and almost undetectable in *Traf6*$^{-/-}$ mice (**Fig. 7a**). In contrast, at this stage, the control littermate thymus showed a progressive increase in Cld3,4$^-$MTS10$^+$ TECs surrounding Cld3,4$^+$ TECs; the numbers of such Cld3,4$^-$MTS10$^+$ cells were also lower (**Fig. 7a**). Nonetheless, substantial numbers of Cld3,4$^-$MTS10$^+$ mTECs eventually developed in postnatal aly/aly and *Traf6*$^{-/-}$ thymi, albeit with sparse distribution among ill-defined cortico-medullary borders in the almost complete absence of Cld3,4$^+$ mTECs (**Fig. 7a**). We confirmed those results by flow cytometry, which showed fewer C-CPE^{hi} UEA-1$^+$ MHC class II–high TECs in the adult aly/aly thymus (**Fig. 7b**). Rare residual Cld3,4$^+$ TECs corresponding to C-CPE^{hi} MHC class II–low UEA-1$^-$

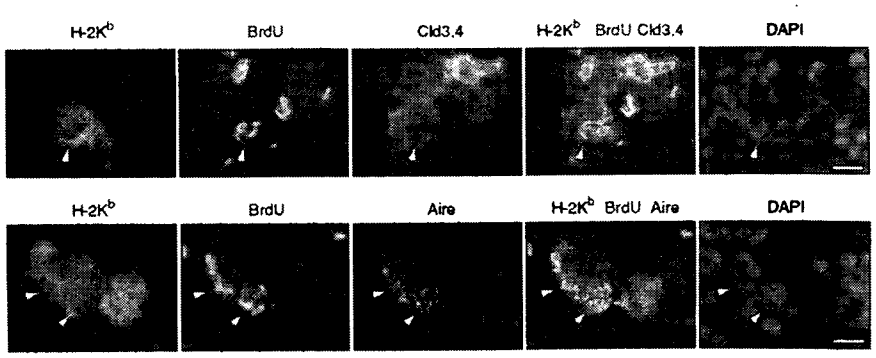

Figure 6 Cld3,4$^+$ Aire$^+$ mTECs arise from C-CPE^{hi} UEA-1$^+$ TECs at E13.5 with cell proliferation. Nude mice grafted with thymic cell aggregates containing the sorted R1 fraction from E13.5 B6 thymic lobes and E13.5 total BALB/c thymic cells were fed BrdU (0.8 mg/ml) for 9 d after the transplantation; 3 weeks later, ectopic thymi were analyzed by four-color immunostaining with DAPI, anti-H-2K^b and anti-BrdU, plus anti-Aire or anti-Cld3,4. Arrowheads indicate donor-derived TECs that incorporated BrdU *in vivo*. Top and bottom rows are different fields of the same thymic section. Scale bars, 10 μm. Experiments were repeated three times with similar results.

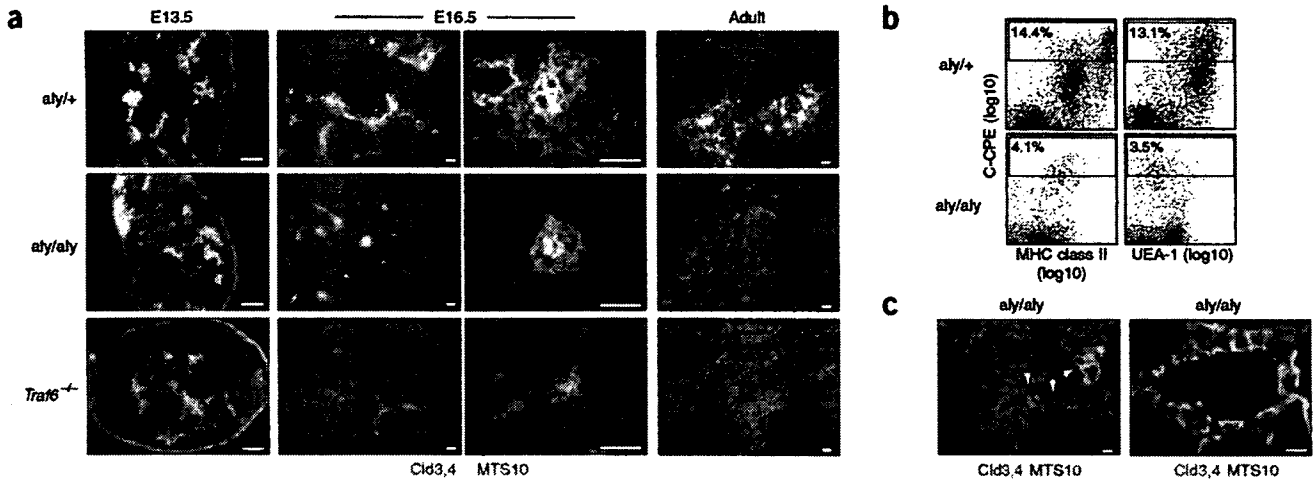

Figure 7 Embryonic development of Cld3,4$^+$ mTECs is profoundly impaired in aly/aly and TRAF6-deficient mice. (**a**) Two-color immunostaining (with anti-Cld3,4 and anti-MTS10) of thymic lobes from aly/aly, aly/+ and *Traf6*$^{-/-}$ mice at E13.5 and E16.5 and postnatally (Adult; 5 weeks for aly/aly and 2 weeks for *Traf6*$^{-/-}$). Data represent merged images only. (**b**) Flow cytometry of thymic stromal cells from 5-week-old aly/aly and aly/+ mice with anti-CD45 and biotinylated C-CPE, plus anti-MHC class II or FITC-UEA-1. Numbers in outlined areas percent C-CPE$^+$ cells for profiles of C-CPE versus MHC class II or C-CPE versus UEA-1 in the CD45$^-$ gate. (**c**) Two-color immunostaining of thymi from 5-week-old aly/aly mice with anti-Cld3,4 and anti-MTS10. Arrowheads indicate cyst-like structures. Scale bars, 50 μm (**a**; **c**, top) or 10 μm (**c**, bottom). At least three embryos at each stage and postnatal mice of each mutant strain were examined with similar results.

cells in the aly/aly thymus often formed cyst-like sheet structures in the medulla, in which Cld3,4 proteins were concentrated sharply at the cell adhesion sites, indicative of tight-junction formation (**Fig. 7c**). These results suggested that the Aire deficiency of aly/aly and *Traf6*$^{-/-}$ mice was due mainly to the defect in the proliferation and/or survival of Cld3,4$^+$ TECs at late embryonic stages.

DISCUSSION

Medullary TECs, through 'promiscuous' gene expression, are uniquely able to display tissue-restricted antigens to thymocytes, thereby pre-empting their actual encounter in the periphery[24,25]. The only molecule known to control such 'promiscuous' gene expression is Aire[9], and Aire deficiency results in autoimmune disease in both human and mice[9,26–29]. The mTECs can directly delete thymocytes expressing TCRs specific for certain types of self antigens[30,31]. Thus, mTECs are crucial in central T cell tolerance at least in part through Aire expression. TECs expressing Aire, however, comprise a small proportion of mTECs[24], and the origin and development of Aire$^+$ mTECs is not well understood.

Here we have demonstrated that the tight-junction components Cld3,4 and occludin had substantial and selective expression in a small proportion of adult mTECs that expressed Aire along with large amounts of MHC class II and costimulatory molecules. It is unlikely that the expression of tight-junction components was a reflection of 'promiscuous' gene expression itself. First, whereas individual tissue-restricted antigens are reportedly expressed in only a very small fraction of Aire$^+$ mTECs[24,32], essentially all the Aire$^+$ mTECs were Cld3,4$^+$. Second, among claudin family members, Aire$^+$ mTECs 'preferentially' expressed Cld3,4 proteins, which were intrinsic to the original pharyngeal pouch endoderm. Third, and most convincingly, Cld3,4$^+$ mTECs were present in similar frequencies in the thymi of *Aire*$^{-/-}$ and *Aire*$^{+/-}$ mice. The expression of Cld3,4 was confined to epithelial stromal cells, and rare Aire$^+$ dendritic cells were Cld3,4$^-$.

A small portion of the Cld3,4$^+$ mTECs, on the other hand, lacked Aire protein. However, mTECs in the adult thymus are constitutively turning over[32], and it has been proposed that the mTECs with high expression of MHC class II and CD80 molecules as well as 'promiscuous' gene expression may represent terminally differentiated cells[7]. Our results indicated that Cld3,4$^+$ Aire$^-$ but not Cld3,4$^+$ Aire$^+$ mTEC populations included cycling cells. Given the observation that a small portion of Cld3,4$^+$ mTECs had a MHC class II–intermediate CD80$^-$ surface phenotype, we suggest that Cld3,4$^+$ Aire$^-$ mTECs may well correspond to transitional mTECs.

In fetal ontogeny, expression of Cld3,4, originally at tight junctions of the pharyngeal endoderm, was sustained selectively in the epithelial cells lined at an apical layer as the anlage became progressively stratified. Such a pattern of claudin expression is similar to that in the epidermis, a typical stratified epithelial tissue[33,34]. In agreement with the disruption of the layer structure after immigration of hematopoietic progenitors, the Cld3,4$^+$ epithelial cells were clustered at the central region of the anlage and began to express MTS10 and UEA-1 ligand, representing the first TECs showing known mTEC markers in ontogeny. The disarrayed Cld3,4$^+$ TECs were then disseminated diffusely in the parenchyma and increasingly became surrounded by Cld3,4$^-$MTS10$^+$ TECs. The medulla develops through the coalescence of as many as 300 medullary regions consisting of a few islets each originating from a single progenitor clone[35]. The number of Cld3,4$^+$ TECs at E13.5 was estimated to be around 1,500 cells per lobe, and thus it is possible that these initial Cld3,4$^+$ TECs may correspond to the clonogenic progenitors forming the medullary islets[35].

We examined the differentiation potential of such Cld3,4$^+$ TECs at E13.5 *in vivo* by grafting the reaggregated cells into nude mice. We confirmed that the sorted C-CPE^{hi} TECs almost exclusively generated mTEC clusters. Furthermore, Cld3,4$^+$UEA-1$^+$MTS10$^+$ TECs at E13.5 'preferentially' generated Cld3,4$^+$MTS10$^-$ mTECs, many of which expressed Aire, with proliferation, indicating the presence of committed progenitors for Cld3,4$^+$MTS10$^-$ mTEC subset. However, the large clusters derived from Cld3,4$^+$UEA-1$^-$ TECs at E13.5 contained both Cld3,4$^+$ and Cld3,4$^-$ MTS10$^+$ mTECs. Because the Cld3,4$^+$UEA-1$^-$ TECs were incapable of generating cTECs and thus lacked common TEC progenitor activity, it seemed likely that the Cld3,4$^+$UEA-1$^+$

mTEC progenitors at E13.5 arose from Cld3,4$^+$UEA-1$^-$ mTEC progenitors rather than directly from putative common TEC progenitors, although it remains possible that Aire$^+$ mTECs can be generated additionally from other immediate progenitors. In either case, our results suggested that the Cld3,4$^+$MTS10$^-$ mTEC subset represented a unique lineage of mTECs that diverged as early as E13.5.

Fetal common TEC progenitors capable of giving rise to both cTECs and mTECs have been unequivocally identified[13,36]. So far, the only marker to identify them is MTS20/24 (refs. 11–13). Our results suggest that common TEC progenitors may be included in the C-CPE^{lo} fraction at E13.5. Unlike C-CPE^{hi} TECs, C-CPE^{lo} TECs generated both cTEC and mTEC clusters, in which Cld3,4$^+$Aire$^+$ mTECs were also included. In the stratified anlage at earlier stages (E10.5–11.5), most TECs reportedly expressing MTS20/24 (refs. 11,12) were C-CPE^{lo}, except for the C-CPE^{hi} TECs lining an apical layer. It remains to be determined whether the C-CPE^{hi} TECs in the stratified anlage also express MTS20/24. Serial immunostaining analysis suggested that the Cld3,4$^+$ epithelial cells lining an apical layer at E11.5 were the source of Cld3,4$^+$ mTEC progenitors, including Cld3,4$^+$UEA-1$^+$MTS10$^+$ cells at E13.5, although Cld3,4 and occludin were apparently involved in tight-junction formation in the Cld3,4$^+$ cells at E11.5 but not those at E13.5 and later. Thus, it is possible that the initial commitment of common TEC progenitors to the mTEC lineage might begin earlier along the stratification process of the anlage.

Aire expression is known to be defective in several lines of mutant mice, including those lacking the lymphotoxin-β receptor, the RelB transcription factor, NF-κB-inducing kinase and the adaptor molecule TRAF6, all of which develop autoimmune diseases[22,23,37–39]. Common features of their thymi include fewer mTECs and perturbed medullary architecture[23,38,40,41]. Our results have shown that although the development and distribution of Cld3,4$^+$ TECs at E13.5 was apparently unaffected in aly/aly and *Traf6*$^{-/-}$ mice, their subsequent proliferation at later stages was profoundly impaired. Although the development of both Cld3,4$^+$MTS10$^-$ and Cld3,4$^-$MTS10$^+$ mTECs was affected, the effect was by far more severe for the former than the latter, and thus the postnatal thymi of these mutant mice showed an almost complete absence of the Cld3,4$^+$MTS10$^-$ mTEC subset with lower yet substantial numbers of Cld3,4$^-$MTS10$^+$ mTECs. In the normal thymus, C-CPE^{hi} mTECs had higher expression of transcripts for RelB than did C-CPE^{lo} mTECs, and it might be suggested that the proliferation and/or survival of the former was more dependent on RelB activation mediated by NF-κB inhibitor kinase and TRAF6 than was survival of the latter. These results suggested that the almost complete developmental defect of the Cld3,4$^+$MTS10$^-$ mTEC subset might principally underlay the defective Aire expression in aly/aly and TRAF6-deficient mice.

It was fairly unexpected that the high expression of tight-junction components was continuously sustained in an mTEC subset even after the loss of layer structure during thymic organogenesis. However, the expression of Cld3,4 and occludin was apparently independent of tight-junction formation in these TECs. Because the transfection of claudins alone is sufficient to induce tight-junction strands in fibroblasts[19,42], it may be possible that tight-junction formation is actively repressed in Cld3,4$^+$ mTECs. Further investigation into the regulation of the expression as well as specific function of Cld3,4 in the TECs may shed light on hitherto unknown functions of tight-junction components in these unique epithelial cells.

METHODS

Mice. The aly/+, aly/aly, BALB/c$^{nu/nu}$ (nude), BALB/c and B6 mice used here were purchased from CLEA Japan. *Aire*$^{-/-}$ and *Traf6*$^{-/-}$ mice have been described[29,43]. All mice were maintained in specific pathogen–free conditions in the animal facility at the Center of Laboratory Animals of Kyoto University.

Antibodies. Phycoerythrin- or allophycocyanin-conjugated anti-CD45 (30-F11) and anti-Ter119 (TER-119); fluorescein isothiocyanate (FITC)–conjugated anti–MHC class II (I-A,I-E; M5; 114.15.2), anti-CD80 (16-10A1) and anti-CD86 (GL1); and purified anti-Ly51 (BP-1; 6C3) were from eBioscience. Phycoerythrin-conjugated anti–MHC class I (H-2Kb; AF6-88.5) and anti–MHC class I (H-2Kd; SF1-1.1), anti-MTS10 (MTS10) and FITC-conjugated anti-BrdU (3D4) were from BD Bioscience. Anti–occludin (71-1500), anti-claudin-3 (34-1700) and anti-claudin-4 (36-4800) were from Zymed. Anti-E-cadherin (ECCD2) was from Takara. Anti-desmoplakin (65146) was from PRPGEN. FITC–UEA-1 (FL-1061) was from Vector Laboratories. Anti–keratin 5 (AF138) was from Covance. Anti–keratin 8 (TROMA-I) was from the Iowa University hybridoma bank. Phycoerythrin-conjugated anti-PD-L1 (1-111) and anti-PD-L2 (122) have been described[44]. Rabbit polyclonal and rat monoclonal antibodies to Aire (B1; 02-5H12-2) have been reported[45,46]. Secondary reagents included phycoerythrin-streptavidin (12-4317-87) and allophycocyanin-streptavidin (17-4317-82; BD Bioscience); Alexa Fluor 488–conjugated goat anti–mouse immunoglobulin G (IgG; A11029), donkey anti–rat IgG (A21208) and goat anti–rabbit IgG (A11034), and tetramethylrhodamine-neutravidin (A6373; Molecular Probes); and indocarbocyanine-conjugated goat anti–rat IgM (112-165-075), donkey anti–rabbit IgG (711-165-152) and donkey anti–rat IgG (712-165-153; Jackson ImmunoResearch).

Preparation of C-CPE. The cDNA plasmid containing the C-terminal fragment (amino acids 184–319) of *C. perfringens* enterotoxin (C-CPE) was fused to a ten-histidine tag at the N terminus. Recombinant C-CPE protein was produced in *Escherichia coli*, was purified as described[21,47] and was biotinylated.

Isolation of thymic stromal cells. Thymic stromal cells were isolated as described[48]. Thymic fragments were digested for 30 min at 37 °C in RPMI 1640 medium containing 0.125% (weight/volume) collagenase D (Boehringer Mannheim) and 0.1% (weight/volume) DNase I (Roche) followed by 0.125% (weight/volume) trypsin (Boehringer Mannheim) and 0.1% (weight/volume) DNase I.

Flow cytometry. Cells were stained with various combinations of antibodies and were analyzed with a FACSCalibur (Becton Dickinson). Cells were sorted with a FACSVantage SE (Becton Dickson).

RT-PCR. RNA was purified from sorted cells with an RNease Mini Kit (Qiagen), followed by cDNA synthesis with SuperScript III (Invitrogen) and semiquantitative PCR analysis with Expand High Fidelity Enzyme mixture (Roche). Primer sets are in **Supplementary Table 1** online.

Immunostaining. Immunostaining was done as described[49]. Thymi were 'snap-frozen' in optimum cutting temperature compound (Sakura), and frozen sections 5 μm in thickness were fixed for 30 min at 4 °C with 95% ethanol followed by 100% acetone for 1 min at 25 °C or were fixed for 3 min at 25 °C with 100% acetone. After being blocked with 1% (weight/volume) BSA in PBS, samples were incubated with immunoconjugates or primary antibodies followed by secondary reagents, then were mounted in Mowiol (CALBIOCHEM) and examined with a microscope (Carl Zeiss). BrdU staining was done as described[32].

Reaggregate culture and transplantation. C-CPE^{hi}UEA-1$^+$, C-CPE ^{hi}UEA-1$^-$ and C-CPE^{lo}UEA-1$^+$ TECs sorted from the thymic lobes of E13.5 B6 embryos (1×10^4 cells) were reaggregated together with total enzyme-digested cells from E13.5 BALB/c thymic lobes (3×10^5 cells) *in vitro* on 0.8-mm membrane filters (Whatman). After 24 h, the reaggregates were grafted under the kidney capsules of nude mice. For BrdU labeling, recipient mice were provided with BrdU (0.8 mg/ml) in the drinking water for 9 d after the transplantation. For labeling of adult TECs, B6 mice were injected intraperitoneally with 1 mg BrdU at 4 h before being killed.

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

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AUTHOR CONTRIBUTIONS

Y.H. principally contributed to general experiments and design; H.F. contributed to flow cytometry; T.K. and Y.C. contributed to TRAF6-deficient mice; H.S. contributed to rat monoclonal antibodies to mouse Aire; M.M. contributed to Aire-deficient mice and discussions; and N.M. provided the overall design of the work.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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REVIEW

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Transcriptional regulation in thymic epithelial cells for the establishment of self tolerance

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Abstract

Thymic epithelial cells (TECs) play pivotal roles in the establishment of self tolerance through critical dialogue with developing thymocytes. Unique actions of two transcriptional regulators within TECs, NF-κB-inducing kinase (NIK) and an autoimmune regulator (AIRE), for the establishment of self tolerance have recently been highlighted by studies using a strain of mouse bearing a natural mutation of the *NIK* gene (*aly* mice) and gene-targeted mice, respectively. Previous studies have demonstrated essential roles of NIK downstream of the lymphotoxin-β receptor (LTβR), which is essential for the development of secondary lymphoid organs; *aly* mice lack all lymph nodes and Peyer's patches because of the defective LTβR signaling. Additional roles of NIK in thymic organogenesis downstream of LTβR, mainly through the developmental regulation of TECs, have now emerged, although the corresponding ligand(s) for LTβR participating in this action have not been fully characterized. In contrast, *AIRE*, a gene responsible for the development of an organ-specific autoimmune disease that demonstrates monogenic autosomal recessive inheritance, contributes to the establishment of self tolerance probably by controlling the expression of self antigens through yet undetermined molecular mechanisms. Thus, it is highly likely that a group of genes control self tolerance within TECs through unique and coordinated actions, and that an understanding of this process would help to unravel the pathogenesis of autoimmune disease.

Key words: autoimmune disease, thymic epithelial cell, NF-κB-inducing kinase, *aly* mice, lymphotoxin, AIRE.

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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 [33]. Clarification of a unifying concept for the mechanisms underlying the development of autoimmune disease has been one of the major challenges of immunological studies. Breakdown of self tolerance is considered to be the key event in initiating the disease process, and an understanding the pathogenesis involved is crucial for developing a suitable therapeutic approach. For this reason, it is essential to know how self tolerance is established within the organized thymic microenvironment.

Physical contact between thymocytes and the thymic stroma is essential for T cell maturation and shapes the T cell repertoire in the periphery [39]. Stromal elements that control these processes still remain elusive. Recently, much attention has been paid to the epithelial-cell component of the stroma (i.e. thymic epithelial cells – TECs), since increasing numbers of mutant and gene-targeted mice bearing structural and/or functional TEC defects, many of which are actually associated with autoimmune disease phenotypes, have been reported. In this brief review, I will focus on two transcriptional regulators within TECs, NF-κB-inducing kinase (NIK) and the autoimmune regulator (AIRE), which play crucial roles in the establishment of self tolerance by maintaining the developmental and functional integrity of TECs, respectively, thereby preventing the development of autoimmune disease (Fig. 1).

NIK

NIK is structurally related to mitogen-activated protein kinase kinase kinase [43] and has been shown to phosphorylate both IκB kinase (IKK)-α and IKK-β, which sequentially activate the downstream IκB proteins necessary for NF-κB activation [40]. The alympho-

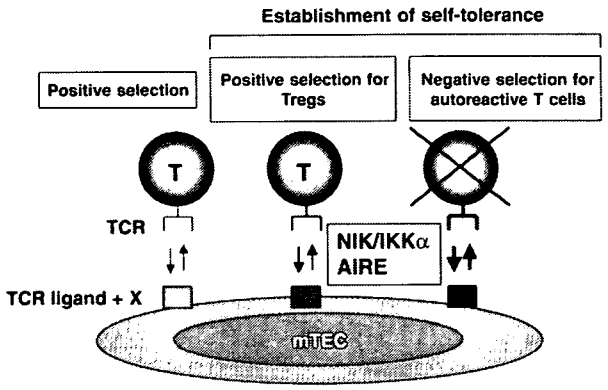

Fig. 1. The transcriptional regulators NIK/IKKα and AIRE within mTECs establish self tolerance through critical dialogues with developing thymocytes. Two known mechanisms of self tolerance, i.e. positive selection for Tregs and negative selection for autoreactive T cells, depending on the avidity of the interactions (depicted by the different depths of shading) between TCR and its self ligands expressed on mTECs are shown. "X" denotes costimulatory pathways and/or undetermined molecules that might additionally contribute to this process.

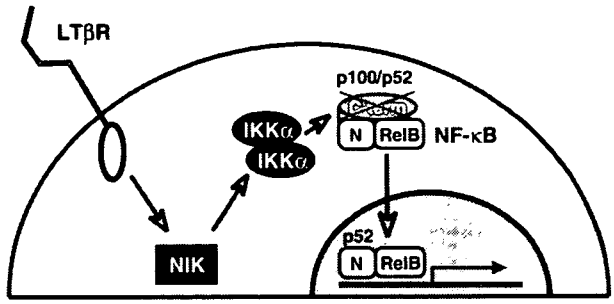

Fig. 2. Alternative NF-κB activation pathway downstream of LTβR within TECs. LTβR ligation activates NIK by yet undetermined molecular mechanisms that result in the activation of IKKα homodimers. Activated IKKα catalyzes p100 of NF-κB2 through ubiquitin-dependent degradation of the C-terminus and generates p52, a mature form of NF-κB2 consisting of the N-terminus. p52 coupled with RelB enters the nucleus to activate the target genes necessary for the differentiation of TECs. *NIK*-mutation in *aly* mice results in inability to bind to and activate IKKα.

plasia (*aly*) strain of mouse carries a natural mutation of the *NIK* gene [47, 57] in which a G855R substitution in the C-terminus of the protein results in an inability to bind to IKKα [46] (Fig. 2). *aly* mice have provided a unique model for the abnormal development of lymphoid organs, since they lack all lymph nodes and Peyer's patches, and the development of spleen architectural features, such as germinal centers and follicular dendritic cell clusters, is disturbed [47, 57, 64]. This is due to defective NF-κB activation through the lymphotoxin (LT)-β receptor (LTβR) [45, 46, 57], which is essential for the development of secondary lymphoid organs [44]. Thymic structure is also disorganized in *aly* mice; the medulla in *aly* mice is smaller than that in control mice, and the boundary of the cortex and medulla is unclear [32, 47, 48, 57]. Importantly, *aly* mice also serve as a model of autoimmune disease, but of unknown etiology [59]; histopathological analysis of *aly* mice has revealed chronic inflammatory changes in several organs, including the liver, pancreas, lung, salivary gland and lacrimal gland [32, 47, 59]. We reasoned that the autoimmune-disease phenotype seen in *aly* mice might be associated with the altered thymic microenvironment. This hypothesis was later proven correct, when thymic chimeras were generated [32]. *aly* mouse thymus-grafted nude mice showed marked lymphoid cell infiltration in the liver and pancreas, accompanied by autoantibody production. In contrast, control thymus-grafted nude mice showed no such changes. Histological evaluation of the grafted thymus revealed that control thymus contained cells reactive with the lectin *Ulex europaeus* agglutinin 1 (UEA-1)$^+$, whereas *aly* mouse embryonic thymus grafted into nude mice did not acquire UEA-1$^+$ cells, suggesting that production of UEA-1$^+$ medullary epithelial cells requires normal NIK in the thymic stromal element.

Although the exact mechanism by which NIK regulates the thymic microenvironment required for the establishment of central tolerance is unknown, the disorganized thymic structure together with reduced *Aire* expression in mice with a mutation disrupting the *RelB* gene merits attention [26]. Because of the phenotypic similarities between *aly* and RelB-deficient mice (multi-inflammatory lesions together with absence of UEA-1$^+$ medullary epithelial cells in the thymus) [62], we speculate that NIK regulates the thymic microenvironment through activation of the NF-κB complex containing RelB. As the production of p52 is impaired in *aly* mouse thymic stroma [32] and NIK has been shown to be necessary for the production of natural killer T cells through the action of RelB [18, 58], it is reasonable to speculate that the NIK-related signaling pathway(s) activates the NF-κB complex in the thymic stroma consisting mainly of p52/RelB heterodimers to generate the thymic microenvironment (Fig. 2).

LTβR and its ligand

Although NIK is an essential component downstream of LTβR [46, 57] and *aly* mice show structural abnormality of the thymus [32, 47, 48, 57], the finding that LTβR-deficient mice have a disorganized thymic structure was somewhat surprising [9] because none of the preceding reports on single-gene knockout mice, either for the membrane-bound LT component (i.e. LTα and LTβ) or LIGHT (two known ligands for LTβR [61]), referred to the thymic phenotypes in these mice [20, 44, 52]. In fact, deficiency of LTα alone was not accompanied by any obvious changes in thymic structure that would have resulted in reduced *Aire* expression at both the transcriptional [32] and protein levels (unpublished observation). LTβR-deficient mice show marked reduction of UEA-1$^+$ cells caused by both loss of the characteristic 3-dimensional organization and a reduction in the absolute number of epithelial cells [9]. In

contrast, LTβ-deficient mice show no significant reduction in the total mass of medullary TECs (mTECs), although changes in the UEA-1$^+$ cell distribution pattern have been pointed out. Introduction of LIGHT deficiency in LTβ-deficient mice resulted in no additional deterioration. Interestingly, LTβ/LIGHT double-deficient mice (lack of both membrane-bound LT and LIGHT) showed less severe thymic disorganization than LTβR-deficient mice, suggesting that LTβR might have additional ligand(s) other than membrane-bound LT and LIGHT [9, 55]. Alternatively, in light of the fact that LTβ-deficient mice show less profound phenotypes of lymph-node genesis (i.e. presence of mesenteric lymph nodes) compared with LTα-deficient mice [3, 36], it is possible that LTα/LIGHT double-deficient mice might show quite equivalent thymic phenotypes to those of LTβR-deficient mice. If this is the case, there may be a weak *in vivo* interaction between secreted LT (i.e. LTα homotrimer) and LTβR [55], although an *in vitro* study did not demonstrate such binding [12]. In either case, it would be important to note that deficiency in LTα (not LTβ) or LTβR seems to result in different thymic phenotypes, even though both deficiencies produce very similar defects in secondary lymphoid organogenesis.

LTβR and autoimmunity

LTβR-deficient mice show some signs of autoimmunity; their serum contains autoantibodies against several organs (i.e. stomach, pancreas and salivary gland) [9]. However, it is not yet clear whether the perivascular accumulation of lymphocytes in multiple organs [20], which is also seen in mice deficient of LTα or LTβ, represents an autoimmune status or an alteration of the lymphocyte distribution caused by defective secondary lymphoid organogenesis. How LTβR signaling controls the thymic microenvironment responsible for the establishment of central tolerance is debatable. One study suggested that LTβR ligation by membrane-LT regulates the transcription of tissue-restricted antigen (TRA) genes (for further discussion, see the section on AIRE) from established mTECs in an Aire-dependent manner [11], whereas another study concluded that LTβR affects mTEC differentiation, thereby controlling TRA gene transcription in the whole thymus, not from individual mTECs [9]. In the latter study, sorted mTECs from LTβR-deficient mice showed indistinguishable levels of both *Aire* and TRA gene expression when equal numbers of mTECs were examined [9]. Consistent with this, mTEC lines established from *aly* mice showed no reduction of TRA gene expression, although total thymus from *aly* mice showed much lower transcription levels of these genes [32, 35]. Thus, a role of LTβR in mTEC development, rather than transcriptional regulation of *Aire* (and TRA gene expression), seems to be a more likely explanation for the autoimmune phenotypes of LTβR-deficient mice. In this regard, it seems important to mention that the spectrum and/or features of autoimmunity are quite different between LTβR-deficient mice and Aire-deficient mice, mirrored by the difference in their autoimmune pathogenesis. An integrated and detailed phenotypic analysis of these mice should help to clarify many aspects of thymus biology.

LTβR signaling and more

NIK-IKKα constitutes an essential component downstream of LTβR for secondary lymphoid organogenesis [46]. It is therefore reasonable to speculate that NIK-IKKα also plays important roles in thymic organogenesis through the action of LTβR signaling. However, given that *aly* mice show more profound thymic reduction and disorganization of mTECs than LTβR-deficient mice [9], it is possible that in this process NIK-IKKα is additionally acting downstream on other receptor(s) beyond LTβR. One hint in the search for such receptor(s) involved in NIK-IKKα-dependent thymic organogenesis is impaired processing of NF-κB2 in thymic stroma from *aly* mice [32] and IKKα-deficient mice [35]. This alternative NF-κB activation pathway [56, 63] was originally demonstrated in hemopoietic cells from *aly* mice [64] and subsequently characterized for LTβR [13] (Fig. 2). Another signal that involves the generation of p52 from a precursor p100 might represent an additional NIK-IKKα-dependent pathway that could fill the gaps of thymic phenotypes between *aly* mice and LTβR-deficient mice.

NF-κB activation within TEC

It is now clear that LTβR/NIK-IKKα is not the only NF-κB-activating axis that regulates thymic organogenesis. Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) has also been demonstrated to be essential for the organization of the thymic microenvironment [1]. TRAF6, an adapter molecule that transduces signals from members of the TNF superfamily and Toll/IL-1 receptor family, activates NF-κB and activating protein 1 [29]. Similarly to *aly* mice and IKKα-deficient mice, TRAF6-deficient thymus-grafted nude mice show marked lymphoid cell infiltration in multiple organs. In contrast to *aly* mice and IKKα-deficient mice, however, NF-κB2 processing in TECs from TRAF6-deficient mice is not impaired. Instead, RelB expression in TECs is severely reduced. TRAF6-dependent RelB expression has been confirmed by the recovery of RelB expression following the introduction of TRAF6 into TRAF6-deficient TECs. Thus, deficiency of NIK/IKKα and TRAF6 merges at the point where p52/RelB complex formation is disturbed, although this does not mean that NIK/IKKα and TRAF6 cooperate together within TECs in order for this heterodimeric complex to be formed. Rather, NIK/IKKα and TRAF6 probably regulate NF-κB activation independently in this process, because TRAF6 deficiency does not affect NF-κB activation downstream of LTβR [1]. The upstream receptor(s) responsible for TRAF6-dependent thymic organogenesis is currently unknown.

AIRE

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 [27]. For this purpose, TECs need to express a set of self antigens (Ags) encompassing all the self Ags expressed by parenchymal organs, a phenomenon termed promiscuous gene expression (PGE) [15, 38]. 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 TRA genes is an autonomous property [15, 39].

Negative selection by Aire

Because Aire-deficient mTECs show reduced transcription of TRA genes [6, 30] and Aire-deficient mice show impaired elimination of autoreactive T cells, as demonstrated with the use of transgenic mice expressing a neo-self Ag under the control of a tissue-specific promoter together with a T cell receptor (TCR) specific for the corresponding Ag [41, 42], it has been postulated that Aire regulates PGE to prevent autoimmunity; mice expressing hen egg lysozyme (HEL) in pancreatic β cells driven by the rat insulin promoter were crossed with mice expressing TCR specific for HEL, and the fate of HEL-specific T cells was monitored in either the presence or absence of Aire. Remarkably, Aire-deficient TCR-transgenic mice showed almost complete failure to delete the autoreactive (i.e. HEL-specific) T cells in the thymus [42]. Because Aire-deficient mTECs showed a reduction in transcription of a group of genes encoding peripheral Ags analyzed by the gene-chip technique [6], it has been hypothesized that pathogenic autoreactive T cells could not be eliminated efficiently due to the reduced expression of corresponding target Ags in the Aire-deficient thymus [41]. However, more recent studies have suggested that some other form of Aire-dependent negative selection is more plausible. Aire-deficient mice develop Sjögren's syndrome-like pathologic changes in the exocrine organs, and this is associated with autoimmunity against a ubiquitous protein, α-fodrin. Remarkably, transcriptional expression of α-fodrin is retained in the Aire-deficient thymus [37].

Because, unlike TRA, α-fodrin is a ubiquitously expressed actin-binding protein [25], one might argue that this finding may not be relevant for the assessment of the Aire-dependent negative selection process. However, subsequent studies using Aire-deficient NOD mice have demonstrated that the mice develop autoreactivity against a prototypic TRA whose transcription is not down-regulated in the thymus [50]. In Aire-deficient NOD mice, acinar cells rather than β-cell islets are the major targets of autoimmune destruction, and this alteration of intra-pancreatic target-organ specificity is associated with the production of autoantibody against pancreas-specific protein disulfide isomerase (PDIp), an Ag expressed predominantly by acinar cells [16, 17]. Transcriptional expression of PDIp is retained in the Aire-deficient NOD thymus, further supporting the concept that Aire may regulate the survival of autoreactive T cells beyond the transcriptional control of self protein expression in the thymus [50]. Conversely, insulin is a prime target Ag recognized by autoreactive T cells in NOD mice [49], and TECs isolated from Aire-deficient NOD mice show reduced expression of insulin. Nevertheless, β-cell islets are relatively less affected by autoimmune attack in Aire-deficient NOD mice [50], which does not support the concept that Aire-dependent transcriptional control of TRA genes in TECs accounts for the development of Aire-dependent autoimmunity. Based on these findings, we suggest that Aire may regulate the processing and/or presentation of self Ags by TECs, possibly through coordinated action with bone marrow-derived cells [21], so that the maturing T cells can recognize the corresponding self Ags in a form capable of efficiently triggering autoreactive T cells. This alternative view of the function of Aire in the establishment of central tolerance has recently been supported by a study with transgenic mouse models [5]. In addition to these models, other Aire-dependent central-tolerance mechanisms as well as self tolerance in the periphery are also possible [53].

Aire in mTEC

Although it is already evident that loss of function of *AIRE* in humans and *Aire* in mice results in the breakdown of self tolerance [51], the physiological roles of AIRE/Aire that implicate self tolerance at the molecular level remain unresolved [2, 24, 60]. From a mechanistic viewpoint, I would suggest two possible models in which Aire exerts its function (Fig. 3). First, Aire plays

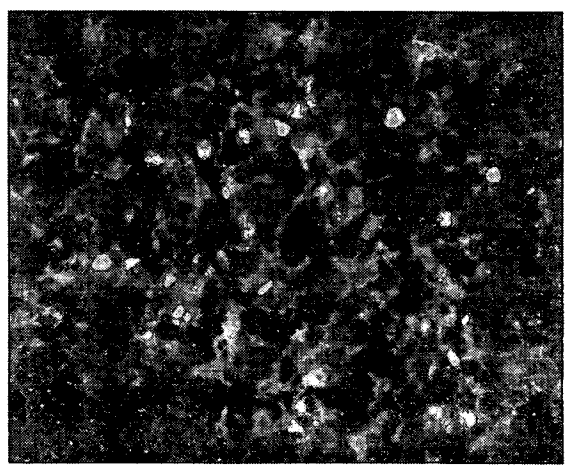

Fig. 3. Aire-positive cells in the thymus. Immunohistochemical staining using antibody against mouse Aire demonstrates Aire-expressing cells (stained in green) scattered in the medullary region of the thymus (stained in red with anti-ER-TR5 monoclonal antibody). Note that Aire is present within mTECs as nuclear dots of protein.

a tolerogenic role within particular type(s) of mTECs, which can be discerned as 'Aire-expressing cells when Aire is present (model 1). In other words, the presence of Aire within the cells is necessary in order for them to function normally as tolerance-establishing cells. In this scenario, Aire-deficient thymus may still contain such cell type(s) but without any tolerogenic function because they lack Aire within them. The second model hypothesizes that Aire is necessary for the development of particular cell type(s), including Aire-positive cells themselves, with tolerogenic function in the thymus (model 2). In this case, we assume that what are called Aire-positive cells and other Aire-dependent cell type(s) do not develop in the absence of Aire. In order to discriminate these possibilities, we 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. If the former model is correct, then Aire-deficiency would not affect the development of phantom Aire-positive cells. In contrast, the latter model would reveal any lack of Aire-dependent cell type(s), again including so-called Aire-positive cells, in the absence of Aire, although extensive immunohistochemical analysis has so far not demonstrated any obvious changes in Aire-deficient thymus [37, 50].

Although both models define Aire-positive cells as a unique cell type among mTECs, we still do not know whether Aire-positive cells are the predominant cell type(s) responsible for the induction of self tolerance. Rather, Aire-positive cells may regulate the function of other tolerogenic mTECs. Similarly, it is not yet clear whether Aire-positive cells are the major source of PGE from mTECs. In fact, a recent single-cell analysis has demonstrated that the expression of Aire in a mTEC is not sufficient for simultaneous co-expression of Aire-dependent TRA genes [23]. Establishment of specific markers for Aire-positive cells may help to clarify these issues as well.

Terminal differentiation model vs. developmental model

One of the key issues for acquiring an understanding of central tolerance is how we accept the dogma that the thymus should and does express a variety of self Ags for the elimination of self-reactive T cells. There are two contrasting models of how mTECs acquire their unique ability to express a broad range of self Ags (i.e. PGE) [22]. The terminal differentiation model assumes that mTECs eventually gain properties of PGE by becoming differentiated, more mature, and more promiscuous [14]. This model suggests that mTECs, especially Aire-positive cells, are specialized cell type(s) that have acquired this property by differentiation. In contrast, the developmental model considers that PGE is a reflection of the multi-potency of immature mTECs before the determination of developmental fate into particular cell type(s) [22]. In this model, the expression of a broad spectrum of TRA genes is considered to be regulated by conserved developmental programs active in developing mTECs. These two models also pose different viewpoints regarding the roles of AIRE/Aire. The terminal differentiation model assumes that AIRE/Aire acts as an important player for the PGE within differentiated mTECs. This is based on the observation that PGE is correlated with CD80 expression levels on mTECs, and that $CD80^{high}$ mTECs from Aire-deficient mice show a reduction in both the total number of overexpressed genes and the relative percentage of TRA genes [14]. In contrast, the developmental model considers that Aire is an important differentiation factor that determines the fate of immature mTECs [22]. As a result, Aire expression does not have to guarantee PGE, as described above [23]. Although not exclusively, the terminal differentiation model and developmental model may respectively favor models 1 and 2 proposed above for the roles of AIRE/Aire.

Regulatory T cells

In addition to negative selection, self tolerance is maintained by another mechanism involving immunoregulatory T cells (Treg) [54], and *Foxp3*, a transcription factor that is genetically defective in an autoimmune disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), is a key regulator for the development of Tregs [19, 28, 34]. So far, no obvious changes in the numbers [6, 37, 42, 50] or function [5, 37] of Tregs have been described in Aire-deficient mice. As a result, Aire/Foxp3 double-deficient mice show fulminant autoimmunity in very early life and a gravely shortened life-span in comparison with mice that are defective in either gene alone [10]. Given that Tregs arise from relatively high-avidity interactions with self-peptide-MHC complexes just below the threshold for negative selection [7, 31, 54], it is somewhat unexpected that Aire deficiency has no major impact on the production or suppressive function of Tregs in both non-autoimmune-prone mice [5, 37] and autoimmune-prone NOD mice [50]. In contrast, *aly* mice [32] and TRAF6-deficient mice [1], both of which show abnormal development of TECs, have reduced numbers of Tregs. The differential requirement of NIK/TRAF6 and Aire for the production of Tregs but the need for all three factors for negative selection, even though impaired negative selection in NIK-mutant mice and TRAF6-deficient mice has not yet been fully demonstrated, might provide a clue as to how the negative selection and production of Tregs are divided, despite the fact that both decisions are dependent on common interactions with TECs, as illustrated in Fig. 1.

CONCLUSIONS

Table 1 shows a general phenotypic comparison between NIK-mutant *aly* mice together with IKKα-deficient mice, and Aire-deficient mice. Even for the two stro-

Table 1. Autoimmune pathogenesis in mice deficient of transcriptional regulators in TECs

	NIK-mutant mice IKKα-deficient mice	Aire-deficient mice
Thymic structure assessed by immunohistochemistry	Defective development of mTECs	Grossly normal
Thymic TRA gene expression detected by RT-PCR	Dramatically reduced in total thymus, but not in individual TECs	Reduced on an individual TEC basis, but not for all TRA genes
Production and function of Tregs	Reduced, but functionally competent	Unchanged
Negative selection studied with transgenic mice	Not assessed, but probably defective	Defective
Possible mechanisms for the breakdown of central tolerance	Defective NF-κB activation required for the development of mTECs	Defective processing and/or presentation of self Ag

mal factors referred to in this article, the exact mechanisms by which they contribute to the establishment of self tolerance still remain unresolved. Although much attention has been paid to the transcriptional regulation of self-Ag expression in mTECs, the precise extent to which its fluctuation might affect the processes of central tolerance per se are still unclear [4]. In this regard, it would also be important to bear in mind that assessment of negative selection using TCR-transgenic mice crossed with transgenic mice overexpressing the corresponding neo-self Ags may not reflect the exact physiological process that contributes to the prevention of autoimmunity [8]. Finally, a more fundamental and persistent issue related to self tolerance is what constitutes the "immunological self" for the immune system. There may be other means by which the thymic stroma achieves expression of the immunological self beyond what I have conceptually outlined in the present article. If this is the case, then the other ways in which T cells achieve recognition of the immunological self might govern the self-non-self discrimination process by as yet additional and unknown rules, similar to the discovery of microorganism recognition by Toll-like receptors, which opened a new era of immunological research.

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