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Temporal Lineage Tracing of Aire-Expressing Cells Reveals a Requirement for Aire in Their Maturation Program

Yumiko Nishikawa,* Hitoshi Nishijima,* Minoru Matsumoto,* Junko Morimoto,* Fumiko Hirota,* Satoru Takahashi,† Hervé Luche,‡ Hans Joerg Fehling,‡ Yasuhiro Mouri,* and Mitsuru Matsumoto*∥

Understanding the cellular dynamics of Aire-expressing lineage(s) among medullary thymic epithelial cells (AEL-mTECs) is essential for gaining insight into the roles of Aire in establishment of self-tolerance. In this study, we monitored the maturation program of AEL-mTECs by temporal lineage tracing, in which bacterial artificial chromosome transgenic mice expressing tamoxifen-inducible Cre recombinase under control of the Aire regulatory element were crossed with reporter strains. We estimated that the half-life of AEL-mTECs subsequent to Aire expression was ~7–8 d, which was much longer than that reported previously, owing to the existence of a post-Aire stage. We found that loss of Aire did not alter the overall lifespan of AEL-mTECs, inconsistent with the previous notion that Aire expression in medullary thymic epithelial cells (mTECs) might result in their apoptosis for efficient cross-presentation of self-antigens expressed by AEL-mTECs. In contrast, Aire was required for the full maturation program of AEL-mTECs, as exemplified by the lack of physiological downregulation of CD80 during the post-Aire stage in Aire-deficient mice, thus accounting for the abnormally increased CD80high mTECs seen in such mice. Of interest, increased CD80high mTECs in Aire-deficient mice were not mTEC autonomous and were dependent on cross-talk with thymocytes. These results further support the roles of Aire in the differentiation program of AEL-mTECs. The Journal of Immunology, 2014, 192: 2585–2592.

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Abbreviations used in this article: AEL-mTEC, Aire-expressing lineage of medullary thymic epithelial cell; BAC, bacterial artificial chromosome; EGFP, enhanced GFP; EpCAM, epithelial cell adhesion molecule 1; mTEC, medullary thymic epithelial cell; tdRFP, tandem-dimer red fluorescent protein; Tg, transgenic; TRA, tissue-restricted Ag; UEA-1, Ulex europaeus agglutinin 1.
Materials and Methods

Mice

A bacterial artificial chromosome (BAC) transgenic (Tg) construct containing 97.1 kb of the 5' region and 69 kb of the 3' region flanking the Aire gene was generated from BAC clone RP23-461E7, in which the Aire start codon was replaced with an open reading frame encoding a tamoxifen-inducible Cre recombinase (17) followed by a poly-A signal from the rabbit β-globin gene. Aire/CreER BAC Tg mice were generated by injecting a linearized BAC Tg construct into pronuclei of fertilized C57BL/6 oocytes, and Tg founders were chosen by Southern blot analysis. A reporter Tg strain expressing enhanced GFP (EGFP) under Cre-mediated recombination (CAG-CAT-EGFP, line 39) (18), a knockin Cre reporter strain expressing a tandem-dimer red fluorescent protein (tdRFP) (19), and Aire/GFP knockin mice (8) were generated as described previously. For induction of Cre recombinase activity, mice were given 500 µg tamoxifen dissolved in 50 µl corn oil i.p. for 6 d. The day after that, on which mice received the final dose of tamoxifen, was counted as day 1. Rag2-deficient mice on a C57BL/6 background were purchased fromTacomic. All mice were maintained under pathogen-free conditions. The protocols used in this study were in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine, Tokushima, Japan.

Immunohistochemistry

Immunohistochemical analysis of the thymus with goat polyclonal anti-GFP Ab (Nobus Biologicals), rabbit polyclonal anti-GFP Ab (Invitrogen), anti–epithelial cell adhesion molecule 1 (EpCAM) mAb (BD), anti-Ly51 mAb (eBioscience), and rat anti-Aire mAb (clone RF33-1) was performed as described previously (8). The mAbs used were anti-CD45 and anti-CD80, both purchased from eBioscience. Ulex europaeus agglutinin 1 (UEA-1) was from Vector Laboratories. Rat anti-Aire mAb was clone RF33-1 prepared in our laboratory.

Results

Characterization of the post-Aire mTEC differentiation program by temporal lineage tracing

Because of Aire expression in the early embryo, it is impossible to perform lineage tracing of AEL-mTECs with a conventional fate-mapping system (15). We sought to overcome this problem by generating BAC Tg mice expressing tamoxifen-inducible Cre recombinase under control of the Aire regulatory element (Aire/CreER BAC Tg), allowing induction of Cre recombinase activity at will (Fig. 1A). One Aire/CreER BAC Tg line was established, and this was crossed with a reporter Tg strain expressing EGFP (CAG-CAT-EGFP) (18) (Fig. 1A) or tdRFP (19) (not depicted in Fig. 1A) upon Cre-mediated recombination. We first performed immunohistochemistry with anti-GFP Ab to monitor GFP expression in the thymus from double-Tg mice 1 d after tamoxifen treatment. We observed many GFP+ cells that were confined to the thymic medulla (i.e., Ly51+ areas) (Fig. 1B), and approximately one- to two-thirds of the cells coexpressed endogenous Aire protein (Fig. 1C). No GFP+ cells were observed in the absence of tamoxifen treatment (data not shown). We also used flow cytometric analysis for detection of GFP signals, together with expression of Aire protein in mTECs at different time points after tamoxifen treatment. Just after tamoxifen treatment, ~30% of the GFP+ cells expressed endogenous Aire, and these cells were almost exclusively from the CD80hi population (Fig. 1D). In contrast, none of the GFP+ cells expressed Aire protein 11 d after tamoxifen treatment, indicating that these cells corresponded to post-Aire mTECs at this later time point. We did not observe any GFP+ cells outside the thymus, including the spleen and lymph nodes, after tamoxifen treatment (Y. Nishikawa and M. Matsumoto, unpublished observations). These results confirmed that we were able to mark the AEL-mTECs reproducibly with this Aire/CreER BAC Tg line to monitor the process of AEL-mTEC maturation.

We then investigated the kinetics of the disappearance of AEL-mTECs from the thymus after tamoxifen treatment by assessing the proportion of GFP+ cells among mTECs on an Aire-sufficient background (Fig. 2A). The proportion of GFP+ cells gradually decreased with time after tamoxifen treatment, and by 3 wk they had almost disappeared from the thymus. Given that transcriptional blockade of the Aire gene in a doxycycline-inducible Aire turnoff Tg system results in the disappearance of Aire+ mTECs over the following 3–5 d (20), it seems reasonable to speculate that the lifespan of Aire protein, once expressed, is < 1 wk. The fact that the total disappearance of AEL-mTECs (GFP+ cells) from the thymus took 3 wk from the initial tamoxifen treatment in our temporal lineage-tracing system suggests that there is a significant post-Aire period (~2 wk) when AEL-mTECs have terminated Aire gene transcription until they finally disappear from the thymus. We estimated the half-life of AEL-mTECs after activation of Cre recombinase activity to be ~8 and 7 d using the CAG-CAT-EGFP (Fig. 2A) and tdRFP reporter strains (Fig. 2C), respectively. It is noteworthy that these estimated times were almost twice as long as those reported previously (i.e., 3–4 d) (10), which would not have accounted for the existence of a post-Aire stage for AEL-mTECs because anti-Aire Ab was used for monitoring.

Dispensable role of Aire in inducing apoptosis of AEL-mTECs

A model has been suggested in which Aire concomitantly induces apoptosis in mTECs, thereby promoting cross-presentation of
TRAs targeted by the transcriptional activity of Aire (10). However, our previous fate-mapping study revealed the existence of a post-Aire stage (15), and our present data show that in fact it is rather long, raising a question about the proapoptotic activity of Aire. To clarify this issue more directly, we compared the survival time of AEL-mTECs from Aire-sufficient and Aire-deficient mice, FIGURE 1.

**FIGURE 1.** Experimental design for the temporal lineage tracing of Aire-expressing mTECs and its specificity. (A) A BAC Tg expressing tamoxifen-inducible Cre recombinase under the control of the Aire regulatory element (Aire/CreER BAC Tg) was generated. After crossing with reporter strains expressing GFP (CAG-CAT-EGFP) or RFP (tdRFP) (not depicted), tamoxifen was given i.p. to facilitate translocation of the Cre recombinase from the cytoplasm to the nucleus to activate the Cre-mediated recombination of the reporter genes. (B) GFP+ cells (green) (left) were observed in the thymic medulla (negative for Ly51, stained red) (center) by immunohistochemistry upon treatment with tamoxifen. Thymi were removed 1 d after tamoxifen treatment. Scale bar, 500 μm. One representative experiment from a total of two repeats is shown. (C) Concomitant expression of GFP (green) and endogenous mouse Aire (red) was examined using anti-GFP Ab (left) and anti-Aire mAb (center), respectively. Scale bar, 20 μm. One representative experiment from a total of three repeats is shown. (D) Endogenous mouse Aire expression from GFP-marked cells was demonstrated by flow cytometric analysis. Thymic stromal cells were isolated enzymatically, and CD45+ GFP+ cells were analyzed for expression of Aire together with CD80 at day 1 (left) and day 11 (right) after tamoxifen treatment. Aire+CD80high cells were detected only at day 1 after tamoxifen treatment among the GFP+ cells. Blue and red dots correspond to CD80high and CD80intermediate to low cells, respectively. Percentages of the cells in the indicated regions are included. One representative experiment from a total of three repeats is shown.
of Aire-expressing mTECs in the absence of Aire was calculated as 8.7 d. An exponential trend line is given, \( R^2 = 0.78 \). Data were accumulated from a total of 10 experiments using 19 mice. ( Compared between heterozygous (i.e., 6.8 d, estimated from Fig. 2C) and an Aire-deficient (i.e., 8.7 d estimated from Fig. 2A) background. Percentages of GFP\(^+\) Aire-expressing mTECs were evaluated from Aire/CreER BAC Tg crossed with CAG-CAT-EGFP, using flow cytometric analysis. Thymic stromal cells were gated for CD45\(^+\) EpCAM\(^+\) UEA-1\(^+\) cells. Each circle corresponds to one mouse analyzed. The half-life of Aire-expressing mTECs in the presence of Aire was calculated as 8.0 d. An exponential trend line is given, \( R^2 = 0.78 \). Data were accumulated from a total of 10 experiments using 19 mice. ( Kinetic properties of Aire-expressing mTECs in the absence of Aire. The half-life of Aire-expressing mTECs in the absence of Aire was calculated as 8.7 d. An exponential trend line is given, \( R^2 = 0.83 \). Data were accumulated from a total of 9 experiments using 13 mice. ( and (D) Aire/CreER BAC Tg mice crossed with the tdRFP reporter mice were further crossed onto Aire/GFP knockin mice. Kinetic properties of Aire-expressing mTEC lineage evaluated at different time points after tamoxifen treatment on an Aire-sufficient (Aire\(^+/gfp\)) (C) and an Aire-deficient (Aire\(^+/gfp\)) background (D). The half-life of Aire-expressing mTECs on an Aire-sufficient and an Aire-deficient background was 6.8 and 6.1 d, respectively. Exponential trend lines are given, \( R^2 = 0.70 \) (for the Aire-sufficient background) and 0.71 (for the Aire-deficient background).

using the temporal lineage-tracing system described above, anticipating that if Aire has any proapoptotic activity, then the lifespan of AEL-mTECs would be prolonged by Aire deficiency within the cells. For this purpose, we further introduced an Aire-deficient background onto Aire/CreER BAC Tg crossed with CAG-CAT-EGFP. It was found that the half-life of AEL-mTECs showed no difference between an Aire-sufficient (i.e., 8.0 d estimated from Fig. 2A) and an Aire-deficient background (i.e., 8.7 d estimated from Fig. 2B), suggesting a lack of any obvious proapoptotic activity of Aire; throughout the observation period, we observed higher percentages of GFP\(^+\) cells among the mTECs in an Aire-deficient background than was the case for those from an Aire-sufficient background. We speculate that this finding may reflect the augmented and/or prolonged transcriptional activity of the Aire locus in the absence of Aire itself, both resulting in higher efficiency of Cre-mediated recombination of the GFP reporter. No obvious alteration in the lifespan kinetics of AEL-mTECs resulting from lack of Aire was demonstrated by crossing Aire/CreER BAC Tg onto the tdRFP reporter strain, in which the effect was compared between heterozygous (i.e., 6.8 d, estimated from Fig. 2C) and homozygous Aire deficiency (i.e., 6.1 d, estimated from Fig. 2D). Thus, Aire itself seems to be irrelevant for efficient cross-presentation through its induction of apoptosis within AEL-mTECs.

Altered differentiation program of AEL-mTECs in the absence of Aire

Alteration of the differentiation program of AEL-mTECs in Aire-deficient mice has been suggested from the phenotypic changes exhibited by the mTECs, including increased numbers of cells with a globular shape (7, 8) and reduced numbers of terminally differentiated mTECs expressing involucrin, in association with reduced numbers of Hassall’s corpuscles (8, 9). However, a more quantitatively noticeable change in the Aire-deficient thymic stroma was an increase of mTECs with a mature signature, expressing CD80 and MHC-II at high levels (10–12). Aire-deficient mice have an increased proportion and/or number of mTECs with high CD80 and MHC-II expression (i.e., mTEC\(^{\text{high}}\)), despite the fact that Aire does not have a direct impact on the division of mTECs, thus leading to the hypothesis that Aire has proapoptotic activity (10). However, the exact mechanism responsible for this phenotype remains unknown. We investigated the mechanisms underlying the possible link between increased CD80\(^{\text{high}}\) mTECs in Aire-deficient mice and alteration of the differentiation program of AEL-mTECs lacking Aire.

We first examined which type of cell, the Aire-expressing or non-Aire-expressing mTEC, is responsible for the increase of CD80\(^{\text{high}}\) mTECs. We used Aire/GFP knockin mice, because this strain allows us to discriminate between Aire-expressing and non-Aire-expressing lineages even on an Aire-deficient background (8). We assessed the expression of CD80 together with GFP after gating for CD45\(^+\) EpCAM\(^+\) UEA-1\(^+\) mTECs. To exclude any gene-dosage effect of the GFP-expressing allele, we made a comparison between mice with the +/gfp (Aire\(^{+/gfp}\)) and −/gfp (Aire\(^{−/gfp}\)) genotypes. As reported previously (10–12) and exemplified in Fig. 3A, the total percentages of CD80\(^{\text{high}}\) mTECs were increased in Aire-deficient Aire\(^{−/gfp}\) mice. Notably, this increase was not observed in the GFP\(^{−}\)/CD80\(^{\text{high}}\) (Aire-expressing) population, but in the GFP\(^{+}\)/CD80\(^{\text{high}}\) (non-Aire-expressing) population (Fig. 3A, Fig. 3B). This finding was rather unexpected because Aire\(^+\) mTECs are a CD80\(^{\text{high}}\) population, and we had anticipated that, conversely, the increased percentages of CD80\(^{\text{high}}\) mTECs would have been made up of cell lineages expressing Aire.

Given that increased CD80\(^{\text{high}}\) mTECs in Aire-deficient mice were made up predominantly of non-Aire-expressing mTECs (including post-Aire mTECs), we suspected that these CD80\(^{\text{high}}\) mTECs from Aire-deficient mice might, for some reason, contain mTECs at the post-Aire stage, which normally exhibit downregulation of CD80 and MHC-II in the presence of Aire (15). Indeed, when the expression levels of CD80 were monitored after tamoxifen treatment in double-Tg mice (i.e., Aire/CreER BAC Tg crossed with CAG-CAT-EGFP), we found that they remained high during the observation period on an Aire-deficient background, whereas they gradually declined on an Aire-sufficient background (Fig. 3C). These results suggested that, in Aire-deficient mice, CD80 is abnormally sustained at a high level during the differentiation program of AEL-mTECs after Aire expression has been terminated (i.e., in the post-Aire stage). A similar pattern was observed when we examined the expression levels of MHC-II at the post-Aire stage (data not shown).

To further confirm that the increase in the number of CD80\(^{\text{high}}\) mTECs in Aire-deficient mice is due to lack of physiological downregulation of CD80 during the post-Aire stage, we applied the tamoxifen-inducible lineage-tracing system to Aire\(^{+/gfp}\) mice,
We investigated whether cross-talk with mature thymocytes is required for the Aire-dependent differentiation program of AEL-mTECs. The program of mTEC maturation is influenced by many factors derived from developing thymocytes (e.g., TNF receptor family ligands, growth factors) (13). We examined whether cross-talk with mature thymocytes is required for the Aire-dependent differentiation program of AEL-mTECs described above. For this purpose, we investigated whether the increase of CD80high mTECs in Aire-deficient mice was due to accumulation of cells, abnormally sustaining their expression of CD80high, even at the post-Aire stage. These results further support our hypothesis that lack of Aire has an impact on the differentiation program of AEL-mTECs.

**FIGURE 3.** Altered differentiation program of AEL-mTECs in the absence of Aire. (A) Thymic stromal cells from Aire-sufficient (Aire+/gfp) and Aire-deficient (Aire−/gfp) mice were isolated enzymatically and evaluated for expression of CD80 and GFP after gating for CD45+EpCAM+UEA-1+ cells. Percentages of the cells in the indicated regions are included. One representative experiment from a total of four repeats is shown. (B) Percentages of total CD80high (left), GFP−CD80high (center), and GFP+CD80high (right) cells were plotted from a total of three experiments. Each circle corresponds to one mouse analyzed. (C) CD80 expression levels from temporal lineage tracing (i.e., GFP+ cells) were monitored at different time points after tamoxifen treatment on both an Aire-sufficient (left) and an Aire-deficient (right) background. Mean fluorescence intensities (MFI) of CD80 were plotted. Each circle corresponds to one mouse analyzed. Gray lines represent mean values. An exponential trend line is given, \( R^2 = 0.72 \) for Aire-sufficient mice and \( R^2 = 0.0009 \) for Aire-deficient mice. Data were accumulated from a total of four experiments.

Cross-talk with mature thymocytes is required for the Aire-dependent differentiation program of AEL-mTECs.
FIGURE 4. Defective physiological downregulation of CD80 on AEL-mTECs at the post-Aire stage in the absence of Aire. (A) Experimental design for the combination of temporal lineage tracing and Aire/GFP knockin mice for evaluation of the stage progression of Aire-expressing mTECs through their differentiation. Aire/CreER BAC Tg mice crossed with the tdRFP reporter were further crossed onto Aire/GFP knockin mice; these were finally crossed onto Aire-deficient mice to generate an Aire-deficient \((\text{Aire}^{+/gfp})\) background (not depicted). Upon treatment with tamoxifen, additional RFP expression onto the currently Aire-expressing GFP\(^\text{+}\) mTECs made the cells GFP\(^\text{+}\)RFP\(^\text{+}\), followed by the GFP\(^\text{-}\)RFP\(^\text{+}\) stage through loss of their ability to express Aire (GFP) at the post-Aire stage. (B) Thymic stromal cells from Aire-sufficient \((\text{Aire}^{+/gfp})\) mice crossed onto temporal lineage-tracing mice were evaluated for expression of GFP and RFP at different time points after tamoxifen treatment to show the stage progression of Aire-expressing mTECs during their differentiation (top). Representative profiles for the expression of GFP and RFP at days 3 and 11 after tamoxifen treatment are shown. Percentages of the cells in the indicated regions are included. Controls at day 3 showing the profiles from Aire/GFP knockin (in which only the GFP signal should be detected) (bottom left) and Aire/CreER BAC Tg mice crossed with the tdRFP reporter (in which only the RFP signal should be detected) (bottom right) for setting the gating windows were also shown. (C) Thymic stromal cells from Aire-sufficient \((\text{Aire}^{+/gfp})\) (top) and Aire-deficient \((\text{Aire}^{-/gfp})\) mice (bottom) were evaluated for expression of CD80 and RFP after gating for CD45\(^\text{+}\)EpCAM\(^\text{+}\)UEA-1\(^\text{+}\)GFP\(^\text{+}\) or CD45\(^\text{+}\)EpCAM\(^\text{+}\)UEA-1\(^\text{+}\)RFP\(^\text{+}\) cells; green (Figure legend continues)
mocytes by generating mice deficient in both Aire and Rag2. The proportions of CD80^high mTECs were much lower on a Rag2-deficient background (Fig. 5), being consistent with the much lower numbers of AEL-mTECs in Rag2-deficient mice (8). We found that the proportions of CD80^high mTECs were indistinguishable between Aire-sufficient and Aire-deficient mice on a Rag2-deficient background, suggesting that the differentiation program of AEL-mTECs may not be absolutely mTEC autonomous with regard to Aire dependency. Instead, cross-talk with mature thymocytes might also play an important role in the Aire-dependent differentiation program of AEL-mTECs.

**Discussion**

Our temporal lineage-tracing approach has enabled us to assess precisely the kinetic properties of AEL-mTECs, including their half-life subsequent to Aire expression (i.e., 7–8 d) and the length of the post-Aire stage (≤2 wk). Our approach has also helped to clarify a number of fundamental and previously unresolved issues related to AEL-mTECs, as follows. We found that Aire plays a neutral role in the induction of cell death among AEL-mTECs, although the roles of Aire in other aspects of cross-presentation per se (e.g., transfer of TRAs from AEL-mTECs to bone marrow–APC and/or the ability of bone marrow–APCs to present TRAs) need to be explored further. We also clarified the mechanisms responsible for the increase of CD80^high mTECs in Aire-deficient mice, which were found to reflect another defect in the differentiation program of AEL-mTECs resulting from lack of Aire.

We have demonstrated that the increase in the number of CD80^high mTECs in Aire-deficient mice was due, at least in part, to lack of physiological downregulation of CD80 during the post-Aire stage. This finding was first obtained by monitoring the levels of CD80 expression in gross lineage-traced cells at different time points after tamoxifen treatment (Fig. 3C). We then introduced the Aire^+/CreER BAC Tg crossed with the tdRFP reporter strain to focus on the genuine post-Aire mTECs (i.e., GFP^+RFP^+ cells in Fig. 4), because GFP^+ cells in the initial analysis might also have included AEL-mTECs still possessing the Aire protein (as exemplified at day 1 in Fig. 1D). Although alterations in the ratios of GFP^+RFP^+ to GFP^+RFP^- cells at different time points justified this experimental system, the lack of any discernible increase of GFP^+RFP^+ cells (from 0.3 to 0.4%) from day 3 to day 11, compared with the significant decrease of GFP^+RFP^- cells (from 1.3 to 0.2%), also suggested a loss of GFP^+RFP^- cells during the assay; post-Aire mTECs might be susceptible to death attributable to their physiological lifetime, and we suspect that some of the GFP^+RFP^- cells might have already died and thus escaped from the analysis, especially at later time points. Obviously, post-Aire mTECs are a heterogeneous population, but the currently available fate-mapping approach does not allow us to discriminate each type of post-Aire mTEC for evaluation depending on the period traced. Accordingly, we analyzed the lineage-traced cells as a homogenous population by changing the time points of observation. We consider that development of novel experimental systems might be required to overcome some of the technical limitations of the current fate-mapping approach, thereby clarifying the dynamic nature of AEL-mTECs individually.

The defective physiological downregulation of CD80 at the post-Aire stage in the absence of Aire, as demonstrated in the current study, together with the reduced numbers of mTECs with mature signatures (8, 9), strongly suggests that Aire is a differentiation-promoting factor rather than one that inhibits the differentiation of AEL-mTECs (6). Given that the lifespan of AEL-mTECs remained unchanged in the absence of Aire, the present results suggest that Aire-deficient AEL-mTECs are lost from the thymus if the maturation signature is incomplete. However, the exact point in the differentiation process at which Aire-deficient mTECs are prevented from differentiating further still remains unclear. Investigation of this issue has been hampered by the current lack of suitable markers for the mTEC differentiation program: so far, CD80 and MHC-II remain the few that are available, and Aire expression may now be added to this profile. Precise elucidation of the target gene(s) relevant to the progression of mTEC differentiation controlled by Aire is an essential task to achieve a full understanding of the roles of Aire in the differentiation program of AEL-mTECs. From a broader viewpoint, future work will need to focus on how the Aire-dependent differentiation program of mTECs contributes to the generation of a tolerogenic thymic microenvironment.

Recently, Metzger et al. (21) developed a similar tamoxifen-inducible fate-mapping system, and confirmed the existence of a post-Aire stage. They found that the half-life of AEL-mTECs was longer than previously thought, as we have demonstrated in the current study. Of interest, their study also showed that Aire^+ mTECs had highly regenerative potential, and that the process depended on RANK signaling, as has been suggested for the production and/or maintenance of Aire^+ mTECs (9, 22). Furthermore, they reported that the spectrum of TRA genes expressed and red dots are from the GFP^+RFP^- and GFP^+RFP^+ populations, respectively. Representative profiles for the expression of CD80 and RFP at days 3 and 11 after tamoxifen treatment are shown. In Aire-deficient (Aire^-/-) mice, a significant proportion of RFP^+ cells remained CD80^high even at day 11. MFIs of CD80 in the indicated populations are included. One representative experiment of a total of three repeats is shown. (D) MFIs of CD80 from GFP^+ RFP^+ populations at days 3 and 11 after tamoxifen treatment on an Aire-sufficient (Aire^+/+gfp^+) (solid circles) and an Aire-deficient (Aire^-/-gfp^-) (clear circles) background. Each circle corresponds to one mouse analyzed. Data were accumulated from a total of 3 experiments using 14 mice.
by post-Aire mTECs was different from that of mTECs with ongoing Aire expression, as suggested previously (9). Thus, the unique properties of post-Aire mTECs in establishing self-tolerance need to be investigated further.

Finally, we found that the increased proportions of CD80\textsuperscript{high} mTECs in Aire-deficient mice were absent on a Rag2-deficient background. The results may suggest that thymocytes (at stages later than double-negative 4), under physiological conditions, provide certain undefined signals that make AEL-mTECs dependent on Aire for their full maturation program. Alternatively, Aire-dependent thymocyte development, as exemplified by the reduced numbers of terminally differentiated single-positive thymocytes in Aire-deficient mice on a Rag2-sufficient background (23), may in turn affect the differentiation program of AEL-mTECs. Thus, some of the unique features of mTECs in Aire-deficient mice are not mTEC autonomous, but cross-talk with mature thymocytes is required for the Aire-dependent differentiation program of AEL-mTECs.

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