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Diversity of TCRs on Natural Foxp3+ T Cells in Mice Lacking Aire Expression

Danielle Daniely,1 Joanna Kern,1 Anna Cebula, and Leszek Ignatowicz

Medullary thymic epithelial cells expressing the Aire gene play a critical role in the induction of tolerance to tissue-specific Ags (TSAs). It was postulated that recognition of Aire-controlled TSAs by immature thymocytes results in the selection of natural CD4+Foxp3+ regulatory T cells (Tregs) and enriches this repertoire in self-reactive receptors, contributing to its vast diversity. In this study, we compared the TCRs on individual Tregs in Aire+ and Aire− mice expressing a miniature TCR repertoire (TCRmini) along with GFP driven by the Foxp3 promoter (Foxp3GFP). The Treg TCR repertoires in Aire+ and Aire− TCRminiFoxp3GFP mice were similar and more diverse than their repertoires on CD4+ Foxp3− thymocytes. Further, TCRs found on potentially self-reactive T cells, with an activated phenotype (CD4+Foxp3−CD62Llow) in Aire− TCRminiFoxp3GFP mice, appear distinct from TCRs found on Tregs in Aire+ TCRminiFoxp3GFP mice. Lastly, we found no evidence that TSAs presented by medullary thymic epithelial cells in Aire−TCRmini mice are often recognized as agonists by Treg-derived TCR hybridomas or CD4+CD25+ thymocytes, containing both natural Tregs and precursors. Thus, positive selection and self-reactivity of the global Treg repertoire are not controlled by Aire-dependent TSAs. The Journal of Immunology, 2010, 184: 6865–6873.
FIGURE 1. CD4+Foxp3+ thymocytes from Aire+ and Aire− TCRmut Foxp3GFP mice express similarly diverse repertoires of TCRs. A, FACS analysis of Aire+ and Aire− TCRmut Foxp3GFP thymocytes. The numbers in the quadrants represent the percentage of cells in the indicated quadrant. Results are representative of at least three independent experiments, each using two or more mice per group. B and C, 2D-F-SSCP analysis comparing the TCRα-CDR3 regions of CD4+Foxp3+ and CD4+Foxp3+ thymocytes from Aire+ and Aire− TCRmut Foxp3GFP mice (total naive spots: 131 versus 145, respectively) (total regulatory spots: 235 versus 232, respectively). The intensity of individual spots corresponds to the real frequency of a particular VαJα rearrangement in the sample. Each spot represents one CDR3α region, except on rare occasions in which more than one sequence may be represented, if their conformation is the same. Nevertheless, the pattern of migration of that sequence would be the same in each population compared. Spots migrating with different speeds on the gel represent different rearrangements. Overlapping spots between two populations are shown in the middle panel colored blue, representing identical VαJα rearrangements, and unique spots are red. Results are representative of three independent experiments, each using two mice per group. D and E, TCRα-
9, 20). Perhaps this inherent self-reactivity allows Tregs to become activated in the absence of foreign Ags, helping them to suppress autoreactive T cells. However, ubiquitously expressed self-Ags are rarely recognized as agonists by TCRs expressed on Tregs, but if TSAs expressed on mTECs select thymic Treg precursors, their self-reactivity could remain concealed in assays utilizing conventional APCs (21, 22). In this report, we examine the impact of Aire-controlled TSA expression on Treg diversity and self-reactivity by analyzing individual Treg TCRs expressed on thymic and nonlymphoid organs, utilizing a novel transgenic mouse model (Aire– mice expressing a miniature TCR repertoire [TCR<sup>mini</sup>], along with GFP driven by the Foxp3 promoter [Foxp3<sup>GFP</sup>]).

**Materials and Methods**

**Mice**

TCR<sup>mini</sup> mice express a polyclonal but restricted set of TCRs. All T cells express the same transgenic VB14-JB2.6 chain but different TCR<sub>a</sub>-chains that form natural rearrangements of the transgenic Va2-Jσ26(Jσ2) mini-locus. TCR<sup>mini</sup> lack endogenous TCR<sub>a</sub>-chains; thus, the diversity of the TCR repertoire is limited to the CDR3<sub>a</sub> region of the transgenic TCR<sub>a</sub>-chain, allowing one to consistently estimate the frequencies of specific TCRs (17). All mice were heterozygous for the TCRs minilocus to ensure expression of a single TCR. The targeted disruption in the Aire gene was designed to mimic the human common autoimmune polyendocrinopathy candidiasis x (APECEx) mutation currently identified (31), and the Foxp3<sup>GFP</sup> mice harbor a bacterial artificial chromosome transgene, which encodes the full locus control region of Foxp3 driving GFP expression (24). All mice were housed under specific pathogen-free conditions in the animal care facility at the Medical College of Georgia (Augusta, GA). All experiments were conducted at the Medical College of Georgia under protocols approved by the Animal Care and Use Committee.

**Cell culture, IL-2 assays, and CD69 expression**

Hybridomas (1 × 10<sup>5</sup>) and thymocytes (1 × 10<sup>5</sup>) were stimulated with either plate-bound anti-CD3 mAbs (2 μg/ml) or purified mTECs (1 × 10<sup>4</sup>) from neonatal thymii. The mTECs were purified by Ficoll after 5 d of culture in the presence of dGuo as described (25). After 24 h in coculture, the supernatant from the hybridomas was evaluated for the presence of IL-2 as described (21). Alternatively, the activation of CD4<sup>+</sup> thymocytes cocultured with mTECs was evaluated by monitoring CD69 expression by flow cytometry.

**Flow cytometry and MoFlo sorting**

Single-cell suspensions were prepared from all lymphoid organs by mechanical disruption through nylon mesh; nonlymphoid organs required mechanical disruption through nylon mesh; nonlymphoid organs required additional digestion with collagenase IV and DNase I. The following Abs were used for flow cytometry analysis and sorting: anti-CD4–APC, anti-CD8–PE-Cy7, anti-CD25–FITC, anti-CD25–PE-Cy5, anti-CD62L–PE, anti-Va2–PE, and anti-Vβ14–FITC. All mAbs used in this study were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Intracellular staining for Foxp3 expression was performed using an intracellular staining kit according to the manufacturer’s protocol (eBioscience).

**RT-PCR and two-dimensional fluorescent single-stranded conformational polymorphism**

RNA was isolated from sorted populations using Trizol according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). cDNA synthesis was done with M-MLV reverse transcriptase (Promega, Madison, WI) and Random Hexamers. TCR<sub>a</sub>-chains were amplified in a standard PCR reaction with Va2-specific primers. A total of 2 μl PCR product was used as a template for a runoff reaction with a fluorescent primer labeled with Cy3 (synthesized by Integrated DNA Technologies, Coralville, IA). The denatured fluorescent products were then subjected to two-dimensional electrophoresis. The first fluorescent products were separated on a denaturing 8% polyacrylamide gel in capillary electrophoresis. Gel noodles were pooled out of capillaries, trimmed, and loaded on a second dimension of nondenaturing MDE (Cambrex, East Rutherford, NJ) slab gel. Fluorescent images were acquired by scanning the slab gel in a Typhoon 9410 imager and with Image Master 5.0 platinum software (Amersham Biosciences, Piscataway, NJ).

**Sequencing of TCRa-CDR3 regions**

cDNA was prepared as mentioned above then amplified by nested PCR with Va2-specific primers. PCR product was ligated into pCRII-TOPO cloning vector and transformed into Mach1 competent bacteria (Invitrogen). Individual colonies were picked, incubated, and amplified with M13 forward and reverse primers. Alternatively, T cells were single-cell sorted onto 96-well plates containing 5 ml RT buffer and 2 U RNasin (Promega). cDNA synthesis was done with M-MLV reverse transcriptase and Random Hexamers (all from Promega). The entire 10 ml cDNA reaction was used for two rounds of nested PCR via PerfectTag polymerase (5 Prime, Gaithersburg, MD) according to the protocol. Products from the second PCR for Va2 were directly sequenced with Va2_287s primer and analyzed as described (21).

**Results**

**Aire deficiency does not affect global Treg diversity in the thymus**

To address the impact of Aire-controlled TSA expression on TCR diversity, we created a mouse model (Aire<sup>−/−</sup> TCR<sup>mini</sup>Foxp3<sup>GFP</sup>) in which we crossed previously described Aire-deficient mice (23) lacking Aire-dependent TSA expression with our existing TCR<sup>mini</sup> mice, harboring a restricted TCR repertoire, allowing the TCR<sub>a</sub>-CDR3 regions of Va2.9-Jσ26(Jσ2) to be used as a specific marker of individual T cell clones. Then we crossed them with Foxp3<sup>GFP</sup> transgenic mice, in which GFP is driven by the Foxp3 promoter, labeling all Tregs (Supplemental Fig. 1) (24). Previous reports demonstrating TCR<sup>mini</sup> mice showed bias in the selection of CD4<sup>+</sup> thymocytes due to the inherent specificity of the original TCR to A<sub>b</sub>; therefore, the proportion of CD4<sup>+</sup> thymocytes is higher than in wild-type thymii (17). FACs analysis and MoFlo sorting of Aire<sup>−/−</sup> and Aire<sup>−/−</sup> TCR<sup>mini</sup>Foxp3<sup>GFP</sup> thymi revealed similar proportions of CD4<sup>+</sup> and CD8<sup>+</sup> single-positive, CD4<sup>+</sup>CD8<sup>+</sup> double-positive, and CD4<sup>+</sup>CD8<sup>+</sup> double-negative as well as gated CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> thymocytes (Fig. 1A), and the total number of cells recovered from Aire<sup>−/−</sup> and Aire<sup>−/−</sup> TCR<sup>mini</sup>Foxp3<sup>GFP</sup> thymi following sorting of CD4<sup>+</sup>Foxp3<sup>+</sup> thymocytes was similar (data not shown), which agrees with a previously published report demonstrating that Aire deficiency in mice does not affect the total number of Tregs (19).

To determine the TCR diversity of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> thymocytes in Aire<sup>−/−</sup> and Aire<sup>−/−</sup> TCR<sup>mini</sup>Foxp3<sup>GFP</sup> mice thymi, we compared their TCR repertoires using two-dimensional fluorescent single-stranded conformational polymorphism (2D-FSSCP) analysis. In this method, the sequences of TCRα-CDR3 regions (referred to as CDR3<sub>α</sub> regions) derived from different subpopulations of T cells are amplified with fluorescently labeled primers and resolved in two-dimensional gel according to the CDR3<sub>α</sub>’s length (first dimension) and then conformation (second dimension). This method is capable of separating >200 spots representing CDR3<sub>α</sub> regions from the most frequently used TCRs.
and provides a snapshot of the core diversity of TCRs analyzed (17). The data acquired can then be compared between different samples to determine their similarity. As shown in Fig. 1B, CD4+Foxp3+ thymocytes found in Aire+ and Aire− TCRminiFoxp3GFP mice had TCR repertoires overlapping at 91%. Likewise, the TCRs expressed by CD4+ Foxp3+ thymocytes derived from Aire+ and Aire− TCRminiFoxp3GFP mice were similarly diverse, but these repertoires overlapped at 71.5% (Fig. 1C). Because the TCR repertoire of Tregs is inherently more diverse than the repertoire of CD4+Foxp3− T cells, this finding is consistent with previous reports showing similar overlaps of ∼70% and 90%, respectively, for thymic and peripheral lymphoid TCR repertoires in Aire+ TCRmini mice (17). To support this finding, we performed single-cell RT-PCR analysis in which we sorted and then compared the most frequent individual CD4+Foxp3− and Treg CDR3a regions from Aire+ and Aire− TCRminiFoxp3GFP mice. The most common CDR3 length is 8 aa.

![FIGURE 2. Dominant TCRs expressed on CD4+Foxp3− and CD4+Foxp3+ thymocytes in Aire+ and Aire− TCRminiFoxp3GFP mice are asymmetrically distributed. A and B, The frequencies of the most dominant CD4+Foxp3− and Treg clones found in Aire+ and Aire− TCRminiFoxp3GFP mice. Shown amino acid sequences represent fragments of TCRa-CDR3 regions, beginning with the third amino acid after the invariant C residue in all TCRAV genes (Y-L/F-C-A-X-1) and spanning the amino acid immediately preceding the TCRAJ motif (2-F/W-G-X-F-G-T). Clones shared between populations are highlighted by gray boxes. C, TCRa-CDR3 region amino acid length distribution of naive and regulatory sequences obtained from single-cell RT-PCR from Aire+ and Aire− TCRminiFoxp3GFP mice. The most common CDR3 length is 8 aa.](http://www.jimmunol.org/)

CD4+Foxp3− and CD4+Foxp3+ thymocytes in Aire+ and Aire− TCRminiFoxp3GFP mice are asymmetrically distributed. A and B, The frequencies of the most dominant CD4+Foxp3− and Treg clones found in Aire+ and Aire− TCRminiFoxp3GFP mice. Shown amino acid sequences represent fragments of TCRa-CDR3 regions, beginning with the third amino acid after the invariant C residue in all TCRAV genes (Y-L/F-C-A-X-1) and spanning the amino acid immediately preceding the TCRAJ motif (2-F/W-G-X-F-G-T). Clones shared between populations are highlighted by gray boxes. C, TCRa-CDR3 region amino acid length distribution of naive and regulatory sequences obtained from single-cell RT-PCR from Aire+ and Aire− TCRminiFoxp3GFP mice. The most common CDR3 length is 8 aa.
Tregs is currently unclear. It was hypothesized that it may be a consequence of the asymmetric distribution of TCRs, selection of Tregs on a different pool of self-Ags, or positive selection of Treg precursors by high-affinity TCRs (4, 9, 20, 26). To determine if this trend is conserved in the absence of Aire-controlled TSA expression in the medulla, we compared CD4 Foxp3A and CD4 Foxp3B CDR3a regions in Aire TCRminIFoxp3GFP mice thymus. A similar asymmetric pattern was observed for Aire+ Aire TCRminFoxp3GFP mice (Fig. 2A, 2B). Secondly, when we looked at the CDR3a region lengths for CD4 Foxp3 T cells and Tregs, the same CDR3a regions were abundant in the Aire+ Aire TCRminFoxp3GFP mice (Fig. 2C). Together, these data revealed that the lack of Aire-controlled TSA expression does not affect positive selection of a highly diverse Treg TCR repertoire in the thymus, and its diversity continues to exceed the diversity of TCRs found on CD4 Foxp3 T cells. Thus, if Aire-controlled TSA expression plays a role in Treg selection, these cells may account for only a nominal fraction of this subpopulation and therefore be hidden among the analysis of central lymphoid organs. Perhaps their existence may be visible in nonlymphoid organs, especially if their tropism depends upon their specificity to the TSAs they were selected upon.

**Aire-deficient thymi select many Tregs that expand in nonlymphoid organs and share identical TCRs with Tregs found in Aire+ mice**

Although Aire TCRminFoxp3GFP mice remain healthy during their lifespan, Aire TCRminFoxp3GFP mice develop organ-specific autoimmunity similar to that of wild-type Aire-deficient mice, marked by circulating autoantibodies and T cell infiltrates (D.L. Daniely, A. Cebula, A.K. Ignatowicz, J.X. She, L. Ignatowicz, manuscript in preparation). It has been shown that wild-type Aire-deficient mice develop mild/moderate autoimmunity, primarily affecting the eye, stomach, salivary gland, lacrimal gland, adrenal gland, and gonads, with little/no autoimmune manifestation in the lung, kidney, or heart (27). In addition, of the 30 genes mostly downregulated by the lack of Aire-controlled TSA expression on mTECs, salivary protein 1 and salivary protein 2 were found to be specific to one tissue, whereas only one gene was downregulated for the lung, cytochrome P450, which had shared specificities for liver and intestine, indicating that Aire plays a major role in tolerance induction toward the salivary gland (27). Therefore, we chose to examine TCRs in the salivary gland, an organ with mild/moderate infiltration, and compare them to TCRs found in the lung, an organ with little/no infiltration in Aire TCRminFoxp3GFP mice (Fig. 3) to determine the impact of Aire-controlled TSA expression on the Treg repertoire in nonlymphoid organs. We sequenced TCRs from the lung and salivary gland of Aire TCRminFoxp3GFP mice, as well as the nondraining lymph nodes, and cross-compared them to the TCRs expressed on thymocytes of Aire TCRminFoxp3GFP mice. One would expect that if Aire-controlled TSAs are involved in the selection of tissue-specific Tregs in Aire+ mice, then those Tregs would be absent in Aire- mice. FACS analysis showed that Tregs were more frequent in Aire TCRminFoxp3GFP mice than in Aire- mice. As shown in Fig. 5A, table). In conclusion, we found that in Aire TCRminFoxp3GFP mice, diseased organs are infiltrated by Tregs expressing a diverse set of TCRs, and many of these TCRs can also be found on Tregs selected in Aire TCRminFoxp3GFP mice thymus, meaning that the selection of these Tregs is likely Aire controlled, TSA independent.

**Tregs rarely recognize self-Ags with high affinity**

It has also been proposed that the Treg repertoire is enriched in self-reactive TCRs as a consequence of either being selected by agonist self-Ags in the context of self-MHC class II complexes, and therefore, they are capable of recognizing self-Ags with high affinity or by being diverted from naive self-reactive thymocytes upon self-Ag recognition (4, 9, 21). To determine if Aire-controlled TSAs are frequently recognized with high affinity as agonists by TCRs expressed on Tregs, we examined the specificities of Treg-derived hybridomas cocultured with autologous mTECs. Considering that established mTEC cell lines poorly express Aire, we used dGuo-treated neonatal thymic cultures to isolate fresh EpCAM+Ag+ and Y3P+ (MHC class II) mTECs (Fig. 5A). In the past, T cell hybridomas have been successfully used to detect low abundant endogenous and exogenous peptides and to study Treg TCR specificity (21, 28, 29). Therefore, we established hybridomas from Tregs isolated from wild-type (C57BL/6), TCRmin, or A Ep single-peptide mice (30) and from CD4 T cells from Scurfy mice. As shown in Fig. 5B, all hybridomas responded

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**FIGURE 3.** Aire TCRminFoxp3GFP mice develop autoimmune manifestations in the salivary gland. Representative histopathology of the salivary glands and lungs in Aire Aire TCRminFoxp3GFP mice (12–15 wk). A and B, Healthy lung (×20) and salivary gland (×10) from Aire TCRminFoxp3GFP mice. C and D, Lung (×20) with little infiltration and salivary gland (×20) showing mild infiltration without tissue destruction. Organs were fixed with 10% neutral buffered formalin, sections were cut at 8 μm and stained with H&E.
FIGURE 4. Many TCRs expressed on CD4+Foxp3+ thymocytes in Aire+ TCRminiFoxp3GFP mice are also found on peripheral CD4+Foxp3+ T cells in Aire- TCRminiFoxp3GFP mice. A, FACS analysis of CD4+Foxp3+ cells in salivary gland and lung of Aire- TCRminiFoxp3GFP mice. B, Cross-comparison of the frequencies of total regulatory TCRs-CDR3 region amino acid sequences in Aire+ TCRminiFoxp3GFP thymus to Aire- TCRminiFoxp3GFP thymus, lymph node, salivary gland, and lung. The table below the graph shows the total and unique/different number of sequences analyzed and shared. The percentage shared of total was calculated by dividing the total shared by the total analyzed; the percentage of unique shared was calculated by dividing the unique shared by the unique analyzed; and percentage of unique of total was calculated by dividing the unique by the total analyzed. Results are representative of three independent experiments using a total of 11 mice to retrieve 1075 regulatory sequences.
to anti-CD3 stimulation. However, only hybridomas derived from A0Ep (these TCRs were not tolerant to naturally processed self-Ags) and Scurfy mice (established from naturally arising self-reactive T cells) responded to self-Ags presented by mTECs, suggesting that TCRs expressed on Tregs rarely recognize self-derived peptides presented by mTECs as agonists. However, Treg-derived hybridomas from TCR mini mice frequently responded when challenged with non–self-Ags and recognized Ags derived from commensal bacteria following adoptive transfer into lymphopenic hosts (21). Therefore, though Tregs do not recognize Aire-controlled self-Ags with high affinity, they can strongly respond to a variety of foreign Ags.

Ithas been proposed that during thymic ontogeny, CD25 expression can precede Foxp3+ expression on CD4+ thymocytes and binding of the TCR to an agonist peptide in the medulla may turn on Foxp3 expression, leading to the final lineage commitment of CD25+Foxp3+ thymocytes to Treg lineage (31). Hence, in the second experiment, we compared CD69 upregulation as a marker of TCR signaling/activation between CD4+CD25− and CD4+CD25+ thymocytes from Aire+ and Aire− mice cocultured with fresh mTECs isolated from Aire+ mice or with immobilized anti-CD3 mAb. Results are representative of two independent experiments with hybridomas using the indicated number of hybridomas each time and three independent experiments for CD69 up-regulation using at least five mice per experiment for mTEC isolation and at least two mice per experiment per group for thymocytes.

FIGURE 5. Self-Ags presented by mTECs in Aire+ mice are infrequently recognized as agonists by Treg cells. A, FACS analysis of dGuO-treated neonatal thymic cultures from Aire+ thymi used to isolate fresh EpCAM+Ab+ mTECs. B, IL-2 secretion/T cell activation assay using hybridomas derived from Tregs isolated from wild-type, TCR mini, and A0Ep single-peptide mice or from autoreactive CD4+ T cells from Scurfy mice. Hybridomas were cocultured for 24 h in the presence of anti-CD3 mAb or with freshly isolated mTECs. C, FACS analysis of CD69 upregulation by CD4+CD25+ thymocytes from A0Ep, Aire+, and Aire− mice cocultured with fresh mTECs isolated from Aire+ mice or with immobilized anti-CD3 mAb. Results are representative of two independent experiments with hybridomas using the indicated number of hybridomas each time and three independent experiments for CD69 up-regulation using at least five mice per experiment for mTEC isolation and at least two mice per experiment per group for thymocytes.

Discussion
In this report, we examined the impact of Aire-controlled TSA expression on the diversity and self-reactivity of TCRs expressed on natural CD4+Foxp3+ T cells in Aire+ and Aire− TCR miniFoxp3GFP mice. The restricted TCR repertoire allowed us to intricately

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compare TCRs at the level of a single T cell clone. One may argue
that the restriction of the TCR repertoire in our mouse model could
unintentionally compromise its diversity, regardless of Aire-con-
trolled TSA expression. Nevertheless, when TCRmini mice were
crossed with autoimmune-prone strains, such as NOD (R. Pa-
cholczyk, personal communication), Aire-deficient, or Scurfy (P.
Kraj, personal communication), it resulted in multiorgan auto-
immunity or death, respectively. In contrast, when TCRmini mice
were crossed with nonautoimmune-prone wild-type C57BL/6 or
129 mouse strains, these mice remained healthy throughout their
life span. Therefore, not only does the restricted TCR repertoire
house sufficient autoreactive TCRs capable of driving the devel-
opment of autoimmune disease, it also houses a sufficient amount
of Tregs capable of maintaining the health of nonautoimmune-
prone mice. In addition, our results show that the CD4+Foxp3+
TCR repertoires in Aire+ and Aire− TCRminiFoxp3GFP mice were
similar and more diverse than their repertoires on CD4+Foxp3−
thymocytes, which were almost identical. Many CD4+Foxp3+
thymocyte TCRs were not found on CD4+ Foxp3− thymocyte
TCRs, whereas the majority of CD4+ Foxp3− thymocyte TCRs
could be found in the regulatory population. Thus, as previously
reported, the asymmetric distribution of TCRs on CD4+Foxp3−
and CD4+Foxp3+ thymocytes is preserved in Aire-deficient mice,
and the diversity of their thymic TCR repertoires is not compro-
mised in the absence of Aire-controlled TSAs.

It has been reported that the selection of transgenic immature
thymocytes by high affinity, self-reactive interactions with MHC/
peptide ligands in the medulla can induce Foxp3 expression and
direct CD4+CD25+Foxp3− Treg precursors toward the regulatory
lineage. Yet, when we tested TCRs derived from Tregs toward
self-Ags presented by thymic medullary epithelial cells, we found
no evidence of abundant, self-reactive TCRs specific for Aire-
controlled TSAs. Additionally, our experiments also show that
CD4+CD25+ thymocytes that contain both natural CD4+Foxp3+
Tregs and their immediate precursors do not express TCRs
capable of recognizing auto-Ags with high affinity on thymic
medullary epithelium. In TCRmini thymii, the relative proportion
of CD25+Foxp3− thymocytes is higher than in wild-type thymii.
We hypothesize that this subpopulation may be enriched in thymocytes that are currently undergoing recruitment, whereas the CD25+Foxp3+ population of thymocytes may represent those that are already committed (Fig. 1A) (24, 31).

It has been shown that self-Ags are involved in the development of tissue-specific Tregs (32). Therefore, our data do not exclude the existence of infrequent Aire-controlled TSA-dependent Treg clones, which may contribute to organ-specific tolerance. However, transplantation of Aire+ fetal thymus into Aire- hosts by another group did not rescue the host from development of organ-specific autoimmunity, which questions the significance of those Tregs (19). In addition, we show that nonlymphoid organs subject to autoimmune manifestation in Aire-deficient mice harbor many Tregs identical to those found in wild-type mice, suggesting that selection and TSA-associated tropism of those Tregs is Aire independent. Also, our study does not account for the occurrence of inducible Tregs that may arise in an autoimmune-prone environment (33). However, the TCRs expressed on the most abundant naive and Treg clones in the salivary gland of Aire- TCRfluorescentFoxp3GFP mice were non-overlapping, suggesting that there is no major recruitment of dominant CD4+Foxp3+ cells to the regulatory lineage.

Our data, along with a previously published report, suggest that perhaps the thymic precursors of Tregs use Aire-independent self-Ags for their selection and lineage commitment, which, in the absence of Aire-controlled TSSAs, similar to self-reactive thymocytes, populate nonlymphoid organs (34). As a consequence, those Tregs and self-reactive thymocytes would express dissimilar TCR repertoires if the former repertoire were chosen independently of Aire and selected within a specific niche, whereas the latter repertoire escaped negative selection as a result of the lack of Aire (7). To determine the similarity between the TCRs of Tregs and those of activated T cells in Aire- TCRfluorescentFoxp3GFP mice, we examined the dominant TCRs on Tregs and activated T cells in the lung and salivary gland and observed little overlap in the lung and none in the salivary gland (Fig. 6C). These results implied that in Aire- TCRfluorescentFoxp3GFP thymi, selection generates Tregs that can specifically accumulate in diseased organs and express TCRs unlike TCRs expressed on activated T cells. However, these Tregs are not sufficient to fully control the development of autoimmunity. Therefore, it appears that the majority of Tregs are not selected by Aire-controlled TSSAs. Thus, although the most anticipated effect of Aire deficiency one would expect is not a quantitative but rather a qualitative compromise of Treg diversity and antigenic specificity, based on our analysis, that appears to be unseen.

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Disclosures

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