Control of Central and Peripheral Tolerance by Aire

by

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Mutations of the Autoimmune Regulator (Aire) were first identified as the cause of a multi-organ autoimmune syndrome, APS-1, in 1997, and it has since become appreciated that Aire normally functions to prevent autoimmunity by facilitating self tolerance to peripheral antigens. Specifically, Aire is expressed at high levels in the medullary epithelial cells in the thymus, the site of T cell development, where it drives expression of otherwise silent, tissue-specific antigens (TSAs) like insulin, resulting in central tolerance towards TSAs. Here, we describe the pattern of Aire expression among medullary thymic epithelial cells (mTEC) and the potential of Aire+ mTECs to recover from targeted ablation. We demonstrate that Aire+ mTECs have a remarkable ability to quickly recover from targeted ablation in a manner that depends on signaling through the TNF superfamily member RANK. We also show that Aire+ mTECs harbor a previously unappreciated potential to reduce Aire and MHCII expression and migrate towards the center of the thymic medulla while maintaining TSA expression. In addition to its role in the thymus, Aire expression has also recently been identified in population of extrathymic Aire-expressing cells (eTACs) residing in the peripheral lymphoid tissues. We address the ability of eTACs to contribute to CD4+ T cell tolerance, and find that eTACs are capable of inducing anergy of autoreactive T through a signaling pathway that depends in part on low expression of costimulatory CD80 and CD86 molecules by eTACs. Finally, we identify eTACs as a non-conventional component of the classical dendritic cell lineage, suggesting that they can be efficiently expanded for future functional studies and potentially for therapeutic induction of tolerance.
Table of Contents

Title page.................................................................................................................................................. i

Abstract........................................................................................................................................................ iv

Chapter I: Control of Central and Peripheral Tolerance by Aire......................................................... 1

Positive selection........................................................................................................................................ 2

Negative Selection........................................................................................................................................ 4

Aire directs tissue-specific antigen expression...................................................................................... 5

Mechanistic control of tissue-specific antigen expression by Aire...................................................... 9

*Aire* expression is restricted to a mature subset of mTECs................................................................. 12

Thymocyte-mTEC interactions are required for proper mTEC maturation.............................. 15

Dendritic cells contribute to presentation of Aire-driven antigens................................................. 19

Aire-directed tissue-specific antigens drive negative selection......................................................... 20

Aire may also promote central tolerance through additional mechanisms.................................. 22

*Aire* expression in peripheral lymphoid tissues can promote tolerance........................................ 25

Peripheral *Aire* expression may complement the function of thymic Aire................................. 28

Aire-independent peripheral tissue-specific antigens......................................................................... 30

Chapter II: Lineage tracing and cell ablation identifies a post-Aire expressing thymic
epithelial cell population......................................................................................................................... 48

Introduction................................................................................................................................................. 49
List of Figures

Chapter I

Figure 1.1. Aire$^+$ mTECs may represent a terminally differentiated TEC population........13

Figure 1.2. Central and peripheral Aire expression promotes tolerance through complementary mechanisms.................................................................31

Chapter II

Figure 2.1. The Aire-DTR mouse facilitates efficient ablation of Aire+ mTECs............52

Figure 2.2. Extended Aire+ mTEC ablation depletes all mTEC subsets.......................56

Figure 2.3. mTEC ablation leads to impaired CD4$^+$ T cell selection and autoimmunity..58

Figure S2.1. Negatively selected OT-II CD4+ T cells undergo TCR rearrangement......61

Figure 2.4. The Aire-Cre mouse allows inducible labeling of Aire-expressing mTECs...64

Figure 2.5. Post-Aire mTECs downregulate MHC Class II.......................................67

Figure S2.2. Post-Aire mTECs have a distinct localization within the medulla and do not contain mTEC progenitor capability..................................................70

Figure 2.6. Post-Aire mTECs lose maturation markers but maintain intermediate TSA expression........................................................................................................73

Figure 2.7. RANK signaling controls Aire+ mTEC development...............................76
Chapter III

Figure 3.1. The AdBDC transgene drives expression of the p31 mimotope peptide in Aire-expressing cells.................................................................100

Figure 3.2. eTACs in AdBDC mice interact with CD4+ BDC2.5 T cells and prevent induction of autoimmune diabetes in SCID mice................................................103

Figure S3.1. Direct antigen presentation by eTACs and anti-DEC1040 treatment both induce robust proliferation of BDC2.5 T cells..................................................105

Figure 3.3. Regulatory T cells are dispensable for eTAC-induced tolerance...............108

Figure S3.2. Treg-depleted BDC2.5 T cells fail to infiltrate the pancreas following antigen-specific interactions with eTACs.........................................................111

Figure 3.4. eTACs induce functional inactivation of cognate T cells by presenting antigen in the absence of costimulation.........................................................114

Figure S3.3. BDC2.5 T cells express tolerance-associated markers following antigen-specific interactions with eTACs.............................................................117

Figure S3.4. eTAC-driven tolerance is resistant to blockade of inhibitory pathways.....120

Figure S3.5. Anti-I-Ag7 attenuates BDC2.5 T cell interactions with eTACs in vitro.....122

Chapter VI

Figure 4.1. eTACs are bone marrow-derived..........................................................146
Figure S4.1. Bone marrow-derived eTACs induce $\delta.3$ T-cell deletion.........................148

Figure 4.2. eTACs are derived from classical DC precursors........................................151

Figure S4.2. Unbiased gating of extrathymic Aire-expressing cells, Fc receptor analysis, and comparison to plasmacytoid dendritic cells.................................153

Figure 4.3. eTACs are a distinct type of antigen presenting cell.....................................156

Figure 4.4. eTACs are present in human lymph nodes................................................158

Figure S4.3. Aire localizes to nuclear speckles in mouse and human medullary thymic epithelial cells.................................................................................................160

Chapter V

Figure 5.1. mTECs dynamically progress through stages of induction and repression of Aire.......................................................................................................................173

Figure 5.2. eTACs drive anergy of CD4+ T cells and deletion of CD8+ T cells.........177
Chapter 1

Control of Central and Peripheral Tolerance by Aire
T cells play a major role in guiding the immune system to specifically recognize and remove infectious agents, while also providing lasting immunological memory of previously encountered pathogens. Their ability to recognize a wide variety of foreign antigens is a result of random recombination events that generate T cell receptors (TCRs) capable of recognizing both self and foreign antigens. While the wide range of foreign antigen specificities generated ensures a nearly unlimited potential to recognize pathogens, the production of self-reactive T cells has the potential to result in debilitating autoimmunity, as seen in experimental conditions in which mice lack critical regulatory pathways due to loss of genes encoding Aire or Foxp3. The classical function of these proteins prevents autoimmunity in healthy organisms at two distinct stages during the development and function of T cells. Central tolerance is established within the thymus by purging self-reactive thymocytes, and thus reducing the propensity for autoreactivity among mature T cells in the periphery. Nevertheless, the incompleteness of central tolerance in removing the mature T cells with self-antigen specificity necessitates additional mechanisms to maintain peripheral tolerance, including further clonal deletion of mature autoreactive T cells in the periphery or active suppression of their activation by regulatory T cell populations.

**Positive selection promotes central tolerance by setting the baseline T cell receptor signaling threshold**

T cells are ultimately derived from hematopoietic precursors that arise in the bone marrow, but unlike their B cell cousins, T cell precursors migrate to the thymus to
undergo maturation. Upon entering the thymus, most T cell precursors make a commitment to the αβ T cell lineage while in the double-negative stage, defined by the lack of expression of CD4 or CD8 T cell co-receptors [1-3]. As lineage commitment progresses, successful TCR β chain rearrangement is first validated through a ligand-independent process, followed by a rearrangement of the TCR α chain and upregulation of both CD4 and CD8 [4, 5]. Expression of αβ TCR heterodimers on the cell surface of developing double-positive thymocytes allows cells with functional TCR complexes to be positively selected by peptide-MHC complexes in the thymic cortex [6]. Double-positive thymocytes with a minimal threshold of reactivity to the particular self-MHC haplotypes in the organism’s genetic background survive; thymocytes failing to express a TCR without this basal self-recognition undergo apoptosis. Survival of positive selection allows thymocytes to make a commitment to either the CD4 or CD8 single-positive lineage.

The importance of creating a TCR repertoire with appropriate signaling thresholds is highlighted by the development of autoimmunity when genetic mutations alter the process of positive selection. Zeta-chain (TCR) associated protein kinase (ZAP-70) is a kinase whose activity is critical for TCR signal transmission [7]. A mutation of this gene was subsequently found to be the causative defect in a spontaneous model of rheumatoid arthritis, the SKG mouse, due to attenuated TCR signaling [8]. This and other ZAP-70 mutants with intermediate function were found to have defects in both positive selection and removal of strongly self-reactive thymocytes through negative selection, as reduced signaling ability first necessitates a stronger positively selecting signal from self-ligands and then reduces sensitivity to deletion by a broader set of self-ligands during negative
selection. Alteration of TCR signaling thresholds in both these settings contributes to the formation of a TCR repertoire that has increased propensity for self-recognition [9, 10].

**Negative selection prevents maturation of thymocytes with strong self-reactivity**

After receiving a positively selecting signal and migrating to the thymic medulla, thymocytes must survive the process of negative selection in which thymocytes bearing strongly self-reactive TCRs undergo apoptosis, thus preventing their maturation and subsequent ability to mount an autoimmune response in the periphery. Negative selection can be observed in experimental systems at both the double-positive and single-positive stages in the cortex and medulla, respectively, depending on the location of the negatively selecting signal [6, 11]. This process serves to reduce the frequency of strongly self-reactive T cells in the periphery. The absence of negatively selecting self-peptide-MHC complexes in the medulla leads to an increase in maturation of autoreactive T cells with the potential to cause autoimmunity [12, 13].

The first reports of tissue-specific antigen transcription resulted from the creation of transgenic mice expressing target genes under control of the rat insulin promoter, although these systems were originally designed to target gene expression to the pancreas. Surprisingly, these transgenes and several others with tissue-specific promoters were activated in the thymic stroma, leading to the deletion of antigen-specific T cells and establishment of tolerance [14]. Other experiments suggested that promiscuous expression of tissue-specific antigens within the thymus and subsequent negative selection of antigen-specific T cells could play a physiological role in the prevention of
experimental autoimmune encephalomyelitis [15]. Later, expression of tissue-specific genes within the thymus was found to be particularly enriched in medullary thymic epithelial cells (mTECs), in contrast to cortical medullary epithelial cells (cTECs), CD11c+ dendritic cells (DCs), and F4/80+ macrophages [16]. Taken together, these studies demonstrated the potential for self-antigen expression within the thymus to mediate deletion of autoreactive thymocytes, and suggested that mTECs may have a particularly important role in this process of guiding the thymocyte repertoire.

**Aire prevents autoimmunity by directing thymic expression of tissue-specific antigens**

Autoimmune polyglandular syndrome type 1 (APS1), which has also been called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a monogenic autoimmune disease characterized by multi-organ endocrinopathy such as hypoparathyroidism or adrenocortical failure, chronic mucocutaneous candidiasis, and ectodermal dystrophies [17, 18]. Though the pattern of inheritance was noted to be monogenic, patients with APS1 develop an array of organ-based autoimmune diseases with significant heterogeneity between individuals. In 1997, two groups identified a novel gene responsible for this condition, the *Autoimmune Regulator (AIRE)*. Sequence analysis suggested that AIRE likely participated in protein-protein interactions and DNA binding based on the presence of conserved domains and clustering of disease-inducing mutation within those domains [19-21]. Later, Aire was found to be expressed within the thymic medulla, suggesting that its mechanism of autoimmune prevention could be to
facilitate immune tolerance within the thymus, perhaps by maintaining normal thymic architecture or regulating interactions with thymocytes undergoing negative selection [22, 23]. Furthermore, the detection of Aire specifically within nuclear speckles suggested that Aire might function by directly regulating gene expression within the thymus [22]. Interestingly, mRNA \textit{in situ} hybridization also showed the presence of \textit{Aire} transcripts within both the lymph nodes and spleen [24], and RT-PCR of peripheral blood showed that human \textit{AIRE} message was detected in CD14+ monocytes and DCs [25].

Two independent strains of Aire-deficient mice were made and found to have multi-organ autoimmunity with lymphoid infiltration of target tissues and serum autoantibodies [26, 27]. While the pattern of autoimmunity was slightly different than the clinical symptoms seen in patients with \textit{AIRE} mutations, the overall pattern of multi-organ autoimmunity was consistent between mice and humans. Like APS1 patients, the autoimmunity was predominantly targeted against endocrine organs. Autoimmunity against the salivary gland and retina was the most penetrant phenotype of \textit{Aire} knockout mice on the C57BL/6 background, and infiltrates increased in an age-dependent manner [26]. Further work showed that pattern of \textit{Aire}-controlled autoimmunity was highly strain dependent [28]. The \textit{Aire} knockout mouse was bred onto the non-obese diabetic (NOD) strain, resulting in the expansion of the autoimmune attack to the pancreas and thyroid gland, and an acceleration of infiltration of targets previously identified in C57BL/6 mice [28]. Also, \textit{Aire} knockout mice on the BALB/c background displayed an intermediate phenotype. Curiously, \textit{Aire} knockout mice on the NOD background were completely protected from diabetes, and pancreatic infiltration was restricted to exocrine tissue [29].
The autoimmunity observed in the *Aire* knockout mouse was found to map primarily to the thymus itself, and not to peripheral tissues. Bone marrow chimerism experiments showed that Aire deficiency precipitated disease when it was absent from the radioresistant stroma, but did not substantially affect autoimmune processes when lacking from the hematopoietic compartment [26]. Similarly, thymus transplant experiments demonstrated that the primary contribution of Aire to the prevention of autoimmunity occurred through its thymic expression [26, 30]. More recently, further insight into Aire’s role in the prevention of autoimmunity has come from the creation of a transgenic mouse in which *Aire* expression is repressed by doxycycline administration [31]. In this study, the authors demonstrated that the presence of Aire was most critical during a perinatal window. Transgenic mice on the NOD background developed the characteristic autoimmunity of *Aire* knockout mice only if Aire was absent before weaning; repression of Aire after 21 days of age did not provoke autoimmunity. Experiments in which Aire was experimentally repressed during recovery from lethal irradiation or antibody-mediated T cell depletion showed the absence of Aire promoted autoimmunity in these settings, thus suggesting that its perinatal requirement is a result of relative peripheral lymphopenia in the perinatal mouse [31]. Alternatively, the observed temporal importance of Aire may more directly reflect decreased thymic output of mature thymocytes as the mice age, a phenomenon may be accelerated in mice on the NOD background due to relatively elevated systemic inflammation [32, 33].

Having demonstrated that Aire prevents autoimmunity primarily through its actions in the thymus, Anderson et al. demonstrated that Aire promotes the expression of a multitude of tissue-specific genes within the thymus, and both *Aire* and its controlled
genes are expressed primarily in mTECs. Comparisons of *Aire* wild-type and knockout mice found that thymic transcription of some previously identified targets including insulin was Aire-dependent, while expression of other targets such as GAD67 was not [26, 34]. The identification of interphotoreceptor retinoid-binding protein (IRBP) as a target of eye-specific autoimmune disease in the *Aire* knockout mice led to further investigation of whether or not expression of an individual self-antigen within the thymus could be responsible for the prevention of autoimmunity to a particular target organ in Aire-sufficient mice [35]. IRBP was expressed within the thymus in an Aire-dependent fashion, and its targeted absence from the thymus through transplantation of IRBP knockout thymi into athymic IRBP sufficient recipients led to IRBP-directed autoimmunity. Thus, the absence of a single self-antigen in the thymus was sufficient to lead to autoimmune disease, highlighting the role of this individual thymic TSA in maintaining tolerance. Later, adoptive transfers and depletion of lymphocyte subsets showed that CD4+ T cells were sufficient to mediate autoimmune disease resulting from lack of Aire activity within the thymus, consistent with defective T cell negative selection in Aire deficient mice [36]. Further examination of Aire-deficient mice has recently led to the identification of odorant binding protein 1a as a target of lacrimal gland autoimmunity [37], myelin protein zero as a target in peripheral neuropathy [38], and vomeromodulin as a target of lung autoimmunity [39]. These findings provide additional support for the importance of individual TSAs in preventing autoimmunity, and in the case of vomeromodulin, show the usefulness of examining autoimmunity in Aire-deficient mice as a tool to identify clinically-relevant autoantigens.
Mechanistic control of tissue-specific antigen expression by Aire

Chromatin immunoprecipitation assays have shown that Aire can directly interact with its target TSAs \textit{in vivo}, but the mechanism by which target specificity is determined and transcriptional activation occurs is still poorly understood [40, 41]. While some investigators have found evidence for direct DNA binding by multimers of Aire, a DNA consensus sequence consistent with the observed activity of Aire in the thymus has not been found [42, 43]. A bioinformatics approach to identifying Aire-induced genes found a broad representation of targets across the genome, with individual targets tending to occur together in clusters [34, 44]. When analyzing transcripts in individual Aire-expressing mTECs by single-cell qPCR, Aire-induced transcripts seem to be present in only a fraction of these cells in a stochastic manner [34, 45]. Aire is thus likely to exert its transcriptional activation by promoting the opening of chromatin, allowing transcription of otherwise-repressed target genes. Such a role is consistent with the previously hypothesized properties of Aire’s individual domains.

Closer examination of the conserved domains of Aire provides some insight into the mechanisms by which it might function. At its N-terminus, a CARD/HSR (caspase-recruitment domain, or homogenously staining region) domain is critical for proper Aire function, and may serve as a site of Aire homodimerization as CARD domain mutants failed to aggregate into characteristic nuclear speckles in an \textit{in vitro} transfection system [46]. Furthermore, studies analyzing the effects of transfected variants of Aire and target antigens in HeLa cells showed that cyclic-AMP response element binding protein (CBP), a transcriptional activator [47], colocalizes with Aire and amplifies the transcriptional activation activity of Aire in a CARD-dependent manner, suggesting that CBP may also
interact with the CARD domain of Aire \textit{in vivo}. These data are consistent with previously reported ability of CBP to interact with Aire and activate transcription [48]. Aire also contains a SAND domain, which contributes to transcriptional activation in other proteins that bear this domain, such as DEAF-1 or the Sp100 protein family [49]. Finally, Aire contains two plant homeodomain (PHD) fingers that contribute to transcriptional regulation by Aire, possibly through recognition of histones bearing silenced methylation profiles at the H3K4 lysine [41, 50]. Mutations in the PHD domains are associated with reduced Aire activity and altered nuclear dimerization. In fact, mice harboring \textit{Aire} mutations in their PHD domain that abolish H3K4 binding were found to have a global dampening in transcription of Aire-induced TSAs [51]. Consistent with reduced thymic TSA expression, these mice also developed characteristic multiorgan autoimmunity.

Though several domains suggest a role in transcriptional activation, the mechanisms of Aire-mediated transcription likely depend on its interactions with other transcriptional complex proteins rather than direct DNA binding. In addition to its domains, Aire contains four LXXLL motifs that are often important for protein-protein interactions. Several Aire-interacting cofactors have been identified, but the relative importance of these cofactors \textit{in vivo} has yet to be established. In addition to the previously mentioned CBP, positive transcription elongation factor b (P-TEFb) may play a role in Aire-mediated transcriptional activation. The proposed function of P-TEFb binding complements that of CBP; while CBP may help direct Aire to sites of repressed transcription, P-TEFb and Aire were shown to associate with the elongation phase of transcription, suggesting that Aire might stabilize this process [52]. DNA-PK, a DNA
repair enzyme, can also interact with and phosphorylate Aire at regions outside of its previously described domains to promote *in vitro* transcriptional activation [53]. Recent co-immunoprecipitation experiments conducted by Abramson et al. during a systematic approach to identify new Aire-interacting proteins found that DNA-PK was the most reliable binding partner of Aire [54]. As further support for the relevance of DNA-PK *in vivo*, Aire-dependent TSAs were reduced in the thymi of mice bearing the SCID mutation among the stromal cells of their thymi. Aire was suggested to increase the frequency of double-stranded breaks as part of its transcriptional initiation, thus necessitating the activity of DNA repair enzyme such as DNA-PK. These observed double-stranded breaks may also be associated with Aire’s proposed pro-apoptotic role [55]. Additional binding partners for Aire were found by co-IP, and functions of many of them can be broadly assigned to the processes of pre-mRNA processing, transcription, chromatin binding, and nuclear export of mRNA, all of which could conceivably enhance the transcription of otherwise repressed TSAs [54]. RNA interference was used to knockdown the function of these novel Aire-interacting proteins *in vitro*, and it was found that several of these proteins did in fact promote the transcriptional activity of Aire. Some of these targets were suggested to have a role in proper Aire nuclear localization; for example, knockdown of Ran binding protein 2 prevented nuclear localization of Aire, and it was instead found to reside in the nucleus. Thus, multiple binding partners of Aire have now been described, and several have been directly shown to influence transcriptional activation by Aire. While some *in vivo* relevance has been demonstrated by experiments with SCID thymi, for example, more work is warranted to clarify the *in*
vivo relevance of these Aire-interacting factors and determine the mechanisms by which they assist Aire-directed TSA transcription in the thymus.

**Aire expression is restricted to a mature subset of mTECs**

Aire was initially found to be present within mTECs, and it has since been determined that its expression is specific to a subset of mTECs expressing high levels of both CD80 and MHC class II (Figure 1.1) [34]. Further experiments used BrdU labeling to determine the proliferative status of mTEC subsets and found that while mTEC\(^{\text{lo}}\) (CD80 low, MHC class II low) and Aire\(^-\) mTEC\(^{\text{hi}}\) (CD80 high, MHC class II high) populations both appeared to be dividing, Aire\(^+\) mTEC\(^{\text{hi}}\) cells were not [55]. Instead, the Aire\(^+\) mTEC\(^{\text{hi}}\) cells were non-dividing and replaced by maturing Aire\(^-\) mTECs as part of a cycle of continual apoptosis and replacement. Consistent with the above cycle, transfected Aire was found to promote apoptosis in vitro in the mTEC cell line 1C6. Furthermore, an experimental system in which Aire\(^+\) mTECs were indirectly depleted as a result of cyclosporine treatment showed that these cells repopulated the thymus at the expense of the mTEC\(^{\text{lo}}\) fraction, suggesting a precursor-product relationship between mTEC\(^{\text{lo}}\) and mTEC\(^{\text{hi}}\) cells [56]. Fletcher et al. also found that the mTEC\(^{\text{lo}}\) population had the highest proportion of MTS24\(^+\) cells, a marker present on immature epithelial cells in the fetal thymus that can give rise to a functioning thymus with cortical and medullary architecture in fetal thymic organ cultures [57]. Nevertheless, this proposed relationship among mTECs has not been conclusively demonstrated, and may be at odds with the finding that transcription factors associated with multipotency, such as Nanog and Oct4,
Figure 1.1. Aire\textsuperscript{+} mTECs may represent a terminally differentiated TEC population. mTECs expressing low levels of CD80 and MHC class II (mTEC\textsuperscript{lo}) appear to differentiate into mTECs expressing high levels of both CD80 and MHC class II (mTEC\textsuperscript{hi}). Aire expression (represented by depiction of its characteristic histological nuclear speckling) is restricted to a non-dividing subset of the mTEC\textsuperscript{hi} subset.
Figure 1.1

mTEC<sup>lo</sup>
CD80 -
Class II -
Aire -

mTEC<sup>hi</sup>
CD80 +
Class II +
Aire -

mTEC<sup>hi</sup>
CD80 +
Class II +
Aire +

Diagram shows the transition from mTEC<sup>lo</sup> to mTEC<sup>hi</sup> with and without expression of CD80, Class II, and Aire.
seem to be expressed among mTECs in an Aire-dependent fashion [58]. More recently, using an Aire-driven Cre transgene and floxed GFP-reporter to mark past and present Aire-expressing cells, Nishikawa et al. found more GFP expression activated by their Cre system than in another transgenic mouse that expressed GFP controlled directly by the Aire promoter [59]. If in fact Aire+mTECs are able to give rise to other mTEC subsets, this may be consistent with the findings of Gray et al., as the absence of proliferation does not strictly prohibit the differentiation of Aire+mTECs into other lineages, and the apoptotic activity of transfected Aire may not reflect its in vivo role.

**Thymocyte-mTEC interactions are required for proper mTEC maturation and Aire expression**

Many signaling components required for normal Aire expression have been identified, but the mechanism responsible for directly activating Aire transcription is unknown. Perturbations of NF-kB signaling can prevent appropriate differentiation of mTECs, resulting in reduced Aire representation within the thymus [60]. For example, lymphotxin β receptor (LTβR) knockout mice develop thymi with a markedly reduced mTEC population, and the lack of UEA-1+ mTECs is even more severe in mice bearing the aly/aly point mutation in NIK, a downstream signaling mediator of lymphotxin activation of NF-κB [61]. Ligands for LTβR were detected on thymocytes by LTβR-Fc staining, and mice lacking the ligands LTβ or LIGHT also showed defective development of the mTEC populations. While Aire itself was not specifically examined in these studies, the authors suggest a functional inhibition of Aire based on the development of
autoantibodies against stomach, pancreas, and salivary gland in LTβR⁻/⁻ mice. However, it was later suggested that these deficiencies were due to indirect effects on Aire through a broad inhibition of proper mTEC differentiation, and it was demonstrated that negative selection in LTβR⁻/⁻ mice proceeded more or less normally in the OT-II-RIP-mOVA model of Aire-dependent negative selection [62].

More recent work has found a role for TNF family receptor-ligand interactions in promoting NF-κB activation and proper mTEC maturation. Both CD40 and RANK were found to be important mediators of mTEC differentiation. The absence of either of the TNF signaling pathways mildly reduced mTEC populations within the thymus, and a combined deficiency of both receptors drastically reduced UEA-1⁺ events within the thymic medulla and nearly abolished thymic Aire expression [63, 64]. RANK signaling, through interactions with RANK-L on lymphoid tissue inducer cells, seemed to be especially important during organogenesis of the thymus, as its absence resulted in marked delay in the appearance of mature mTECs. CD40, on the other hand, was postulated to have a more prominent role in maintaining mTEC populations in the maturing thymus in response to CD40L signaling coming from maturing thymocytes. The findings of these two studies, together with the data showing LTβR signaling promotes proper mTEC differentiation suggest that thymocytes deliver multiple signals to enforce proper mTEC differentiation. In fact, it has recently been shown that a reduction in mTEC cellularity, including Aire⁺ mTECs, occurs in response to cyclosporine mediated thymocyte ablation [56].

Sin is a signaling component that has recently been identified as another critical regulator of mTEC differentiation. Within the thymic stroma it is expressed most highly
in mTECs, and its absence in thymic stroma leads to their improper maturation and subsequent deficiencies in Aire expression and immune tolerance [65]. The decreased immune tolerance in these mice was suggested to result from defective negative selection, as OT-II TCR transgenic thymocytes in Sin−/− mice failed to be properly negatively selected in response to RIP-mOVA produced antigen. The authors also found that the global architecture of Sin−/− thymi was altered; there was an increased proportion of mTEC islets within the thymus, suggesting a failure to properly to fuse into a distinct thymic medulla as is normally seen in thymic development. Their final observation in support of a role of Sin in mTEC maturation was the observation that Sin deficiency preferentially impacted the more mature mTEC^hi population, as opposed to the mTEC^lo cells. This finding of a NF-κB independent signaling pathway with relevance to mTEC differentiation should lead to a greater understanding of the control of mTEC development, and elucidation of the Sin signaling pathway may provide new insight into important signaling events between thymocytes and TEC populations. White et al. have also provided fresh evidence that thymocytes themselves promote proper differentiation and function of mTECs, including Aire^+ mTECs [66]. Specifically, using an inducible ZAP70 T cell receptor signaling component, the authors show that T cell signaling and subsequent maturation of thymocytes is required for normal development of Aire^+ mTECs, and that lymphotoxin signaling is likely a component of this communication between mature thymocytes and mTECs. These findings echo previous work by Irla et al. showing that the mTEC compartment fails to develop normally in the absence of CD4^+ thymocytes [67].
While proper thymocyte interactions are important for regulating correct development of the thymic epithelium, Aire might also play a more direct role in its own regulation. An increase in the number of mTECs was noted in Aire knockout mice, and has subsequently been confirmed with the use of a transgenic mouse in which GFP is driven under control of the Aire promoter [26, 68] (Metzger et al. unpublished observations). Other experiments have suggested that Aire knockout mice may have thymi with mildly altered medullary architecture [58]. An Aire-GFP knockin mouse, where GFP was inserted and interrupts the Aire locus, was used to show that Aire knockout mTECs themselves may have differences in their architectural characteristics in comparison to Aire-sufficient mTECs, including increased representation of GFP-expressing mTECs in the Aire knockout mouse [69]. The mechanism behind the observed differences in the frequencies of Aire-expressing mTECs between Aire wild-type and knockout mice remains unknown. While Aire may directly interact with factors controlling mTEC differentiation, and perhaps contribute to a negative-feedback back loop controlling the development of Aire-expressing mTECs, the observed changes in Aire-expressing mTEC frequencies could also be an indirect result of Aire’s control of tolerance; Aire knockout mice develop autoimmunity in peripheral tissues, and increased inflammatory cytokine levels in the circulation might influence thymic microarchitecture. In summary, Aire seems to play a role in its own regulation, although it is not clear whether this role is direct or indirect, and it remains to be determined how Aire itself collaborates with thymocyte-derived signals and other unknown factors to regulate levels of Aire expression within the thymus.
Dendritic cells contribute to presentation of Aire-driven antigens

The major antigen-presenting cells (APCs) within the thymus include mTECs, cTECs, and DCs. cTECs reside in the cortex of the thymus and are primarily involved in providing positively selecting ligands to double-positive thymocytes. Expression of many TSAs occurs only in Aire-expressing mTECs, and, while mTECs synthesize and display high levels of MHC class I and class II complexes, they are not necessarily responsible for directly interacting with and inducing deletion of self-reactive thymocytes. Rather, mTECs could serve as an antigen reservoir, with DCs playing the major role in directly presenting antigen to developing thymocytes. Using RIP-mOVA mice that express the model antigen ovalbumin (OVA) in the thymus, it was found that OVA-specific CD8⁺ T cells underwent thymic deletion even in the absence of class I expression among bone-marrow-derived (APCs) [70]. Conversely, the authors also found that CD4⁺ T cells with OVA-specificity required bone-marrow-derived APCs for thymic deletion, suggesting that mTECs can directly mediate negative selection of CD8⁺ but not CD4⁺ single-positive thymocytes.

More recently, antigen handoff from mTECs to DCs has been directly demonstrated. Using a MHC class I promoter to drive expression of OVA tagged with a nuclear localization sequence, the authors found that while mTECs transcribed the transgene most efficiently, the thymic DCs displayed the strongest stimulatory capacity, suggesting a handoff of antigen from mTECs to thymic DCs [71]. The need for mTECs to transcribe the antigen and hand it off for maximum T cell stimulation was confirmed by using bone marrow chimeras to restrict transcription of the antigen to the radioresistant mTECs. The finding was also extended to endogenous antigens,
confirming the relative strengths of mTECs and DCs to transcribe and present antigen, respectively. Finally, Foxn1-GFP mice expressing GFP in thymic stroma were used to visualize the transfer of membrane bound protein from mTECs to DCs, and mismatched MHC bone marrow chimeras were used to suggest that MHC complexes could be directly transferred from radioresistant mTECs to hematopoietically-derived DCs.

**Aire-directed tissue-specific antigens appear to promote tolerance primarily by serving as ligands for negative selection**

After discovering that Aire directs expression of self-antigens within the thymus and prevents autoimmunity, the next step was to determine how self-antigen expression was accomplishing disease prevention. Negative selection of thymocytes specific for Aire-directed TSAs and induction of regulatory T cells responsive to these self-antigens were two potential ways by which this might be accomplished. Regulatory T cells (Tregs), the majority of which are generated within the thymus as natural Tregs, are required to control autoreactive T cells in the periphery; their absence leads to lethal autoimmunity [72, 73]. Furthermore, Tregs seem to depend on the presence of self-antigen recognition for thymic development. TCR transgenic mice fail to develop Tregs when the absence of RAG recombinase prevents thymocytes from rearranging endogenous TCRs, which, when allowed to occurs, can result in recognition of self-ligands through expression of a second TCR chain [74, 75]. Furthermore, Tregs appear to have a higher propensity for recognition of self-ligands than conventional T cells [76]. Also, their development is promoted by thymic expression of cognate ligand in a transgenic system using
hemagglutinin as a model antigen [77], thus suggesting that the self-antigens whose transcription is directed by Aire in the thymus might also serve as $T_{\text{reg}}$-inducing ligands.

To address the question of how Aire expression promotes tolerance, Hen egg lysozyme (HEL), a model antigen, was expressed in the thymus using the insulin promoter, and Aire’s affect on the selection of antigen-specific T cells was examined in HEL-specific TCR transgenic mice expressing the neo-self-antigen [78]. In this system, negative selection of cognate T cells was dependent on Aire expression, but roughly equivalent numbers of CD25$^+$ $T_{\text{reg}}$s were generated regardless of the presence of Aire. Similar results were also seen in the ovalbumin model antigen system, in which OT-II OVA-specific T cells undergo Aire-dependent negative selection in response to the RIP-mOVA transgene [79]. The authors also found that Foxp3$^+$ CD25$^+$ ratios in the thymus and periphery do not significantly change in Aire knockout mice with polyclonal TCR repertoires. Furthermore, co-transplantation of Aire wild-type and knockout thymi into athymic nude recipients still resulted in autoimmune disease, which could be prevented if a primary role of Aire wild-type thymi is to induce $T_{\text{reg}}$s that dominantly maintain tolerance. Recently, experiments in mice with a Foxp3-promoter-driven GFP reporter and a restricted TCR repertoire suggested that TCR usage by Foxp3$^+$ cells did not significantly differ between Aire sufficient and deficient mice, further supporting the observation that Aire driven antigens do not play a large role in shaping the TCR repertoire [80].

Providing further evidence for the role of Aire in providing negatively selecting ligands, Liston et al. found in two independent model antigen systems that Aire-induced transgenic antigens were less efficiently transcribed among Aire$^{+/-}$ mice than their wild-
type counterparts, and that the heterozygous mice experienced an intermediate level of
cognate T cell negative selection in comparison to Aire wild-type and knockout mice
[81]. This dose-dependent effect of Aire on its target antigens was later confirmed by the
creation of a mouse with a point mutation in the SAND domain of Aire. The mutant Aire
causd a reduction in Aire activity in a dominant negative manner, which led to a milder
form of autoimmunity than that observed in the complete Aire knockout mouse [82]. The
finding that the amount of negative selection occurring to Aire-driven antigens is
proportional to the activity of Aire further support negative selection as the primary
mechanism by which Aire promotes central tolerance.

**Aire may also promote central tolerance through additional mechanisms**

While the above work demonstrated that Aire is important for negative selection
and that global defects among peripheral T$_{reg}$ populations do not result from a lack of
Aire in the thymus, it has not been demonstrated that Aire-induced TSAs cannot
contribute to T$_{reg}$ selection. On the contrary, transgenic expression of an Aire-driven neo-
self antigen was shown to provide positively selecting ligands to support the formation of
natural regulatory T cells which would otherwise not encounter any positively selecting
antigen in the thymus [83]. More recently, Hinterberger et al. provided evidence for a
role of Aire-expressing mTECs in guiding natural T$_{reg}$ development in a polyclonal
setting [84]. Using a bacterial artificial chromosome approached to drive a transgene
under control of the Aire promoter, the authors directed expression of a siRNA that
inhibited expression of the MHC Class II transactivator (CIITA), a master regulator of
MHC class II expression. The resulting mice had a partial reduction in CIITA target expression among mature mTECs, and the strongest target knockdown was observed in the subset of mature mTECs that also express Aire, resulting in an Aire+ mTEC population that maintained its TSA transcription activity but had a reduced surface expression of MHC class II molecules. The result of this deficiency was an increased representation of CD4+ single-positive thymocytes, and an increased ratio of Foxp3+ cells among that fraction. Furthermore, using two systems of Aire-driven antigen and cognate TCR transgenes, negative selection was shown to be impeded by CIITA siRNA, while T_{reg} induction was increased. Therefore, while this work is consistent with the well-known role of Aire+ mTECs in mediating negative selection, it also suggests that antigens presented by Aire+ mTECs affect the induction of T_{regs}, possibly by affecting their negative selection. This possibility was recently confirmed with the identification of Aire-dependent regulatory T cells that develop in a polyclonal repertoire are specific for a tumor antigen [85].

Providing further contrast with experimental systems which identify a role of Aire-driven antigen expression in providing negatively selecting ligands to cognate T cells, autoimmunity against α-fodrin, a ubiquitously expressed actin-binding protein, was observed in Aire-deficient mice, suggesting a lack of proper negative selection despite finding equivalent thymic expression of this antigen between wild-type and knockout mice [30]. A similar result was obtained in response to RIP-mOVA directed antigen expression; reduced negative selection was observed in the Aire-deficient context despite a maintenance of detectable transcript levels [79]. These findings suggest that, in addition to its role in promoting transcription of tissue-specific antigens (TSAs), Aire
may control the process of antigen presentation at a post-transcriptional stage, and may also affect negative selection through ligand-independent pathways. In fact, the work of Anderson et al. in 2005 suggested a list of chemokine receptors and molecules involved in antigen processing events with different expression between Aire sufficient and deficient mTECs [79]. Consistent with the above microarray data, non-transgenic mTECs from Aire wild-type and knockout mice were loaded with exogenous SIINFEKL peptide, and mTECs lacking Aire exhibited a reduced ability to stimulate proliferation among cognate OT-I CD8+ T cells.

Another suggestion that Aire’s role in promoting central tolerance involves more than driving negative selection comes from investigation of human APS1 patients. These patients with AIRE mutations appear to diverge from their mouse counterparts with regard to the importance of Aire in controlling the Treg pool, as two studies suggest that APS1 patients may in fact have defects within their populations of Tregs. In an analysis of APS1 patients and controls, it was found that proportion of circulating CD25+ T cells was the same between conditions, but that the APS1 patients’ CD25+ T cells had impaired suppressive ability in vitro [86]. Looking specifically at Foxp3 expression, it was found that T cells from control patients had in fact 2-fold higher expression levels than samples from APS1 patients, and thus defective Treg induction in human APS1 patients was offered as an explanation for the more severe disease manifestations in patients with defective AIRE, in comparison with knockout mice. A later study also found a relative deficiency in Foxp3+ Tregs resulting from AIRE mutations in humans, and further described this phenomenon as being due to an increased conversion of naïve Tregs that had recently emigrated from the thymus to an activated phenotype, and corresponding
inability of these Tregs from APS1 patients to fully upregulate Foxp3 during activation [87]. The authors conclude that altered Treg ratios in humans are a result of a response to autoreactivity through altered Treg homeostasis in the periphery, and not thymic selection events. However, due to the inherent limitations of studying human disease, these studies do not conclusively rule out altered induction of natural Tregs in the thymus.

**Aire expression in peripheral lymphoid tissues can promote tolerance**

While the role of Aire in contributing central tolerance is well established, its role in secondary lymphoid tissues has been less well known. Initial investigations of Aire expression showed that it was present at the transcript level in both lymph nodes and spleen [22, 26]. This finding was challenging to confirm at the protein level (86), but the creation of a BAC transgenic mouse in which the Aire locus was used to drive GFP facilitated the identification of Aire protein in both lymph nodes and spleen in its characteristic nuclear speckles [68]. The level of protein expression was consistent with differences in Aire transcript; its expression was lower in peripheral tissues, both by immunofluorescence and flow cytometry [68]. Aire was reported to be undetectable in the periphery in mice on the C57BL/6 background [88], while the peripheral Aire observed by Gardner et al. used mice on the autoimmune prone NOD background, suggesting that these different outcomes could be due differences in strain background. However, we have found equivalent activation of Aire-driven GFP among transgenic mice between mice on the C57BL/6 and NOD backgrounds, and were able to detect
nuclear Aire protein by immunofluorescence in C57BL/6 with the aid of the Adig GFP reporter (Metzger et al. unpublished observations).

The transgenic Aire reporter created by Gardner et al. contained not only GFP for the purposes of identifying Aire-expressing cells, but also a type I diabetes autoantigen, islet-specific glucose-6-phosphatase-related protein (IGRP) [68]. This allowed for the determination of whether or not extrathymic Aire-expressing cells (eTACs) could present antigen to cognate T cells, and whether or not such interactions had functional consequences. Adoptive transfer of cognate 8.3 T cells revealed that these cells did indeed proliferate in all secondary lymphoid organs in response to transgenic antigen expression, and that such proliferation ultimately led to their deletion. Furthermore, the ability of eTACs to directly interact with cognate T cells was demonstrated by two methods. First, bone marrow chimerism experiments in which class I was removed from hematopoietically derived cells, including DCs, demonstrated that direct interactions between cognate T cell and radioresistant eTACs could result in T cell proliferation and deletion. Second, two-photon imaging experiments allowed direct visualization of specific, long-lasting contacts between cognate T cells and antigen-bearing eTACs. While not strictly ruling out the possibility that DCs or other hematopoietically-derived components may also participate in peripheral Aire-driven antigen expression and/or presentation, these results show that presentation of endogenously derived antigen by radioresistant eTACs is sufficient to mediate deletional tolerance.

The potential ability of these eTACs to tolerize peripheral T cells through process of deletional tolerance is analogous to the removal of self-reactive thymocytes through negative selection in the thymus. This prompted the question of which signaling
pathways other than specific antigen signals might be relevant to the observed phenomenon, and expression of a panel of important costimulatory molecules by these Aire-expressing cells was analyzed. Flow cytometry revealed that, unlike their thymic counterparts, eTACs surprisingly lacked both CD80 and CD86, surface markers required to provide an activating costimulatory signal through the CD28 receptor on T cells undergoing activation. Like Aire+ mTECs, eTACs expressed both EpCAM and the inhibitory PD-L1 costimulatory ligand. Finally, eTACs in the lymph nodes and spleen displayed high levels of ICOS-L, while mTECs did not, suggesting that this costimulatory ligand may play a role in guiding the outcome of T cell-eTAC interactions. Also, immunofluorescent staining was conducted to determine whether Aire was present among previously identified stromal or hematopoietic fractions of the secondary lymphoid tissues. eTACs were reported to be negative for the B cell marker B220, the fibroblastic reticular cell markers gp38 and ER-TR7, and the DC marker CD11c [68]. Interestingly, eTACs were found to have high levels of MHC class II, suggesting that they may also have a functional capacity to interact with CD4+ T cells, and, because of the relative restriction of class II expression to cells of the immune system, that eTACs are likely to have a fundamental role in contributing to immune regulation. Future experiments, described in Chapter 4, address whether or not eTACs are capable of inducing tolerance in CD4+ T cell populations, and, if so, whether such tolerance involves the processes of clonal deletion, anergy induction, and/or Treg induction.

Recently, Poliani et al. have shown that similar to the findings in mouse, AIRE is detectable at the protein level in humans [89]. In contrast to the mouse findings, AIRE was only detected in the lymph nodes, and not the spleen. A closer examination of
Peripheral *AIRE*-expressing cell frequency also found that abdominal lymph nodes such as the mesenteric lymph nodes had more $AIRE^+$ cells than the more superficial inguinal lymph nodes, for example, likely reflective slight differences in inductive signals between the different environments. Surface marker analyses showed some overlap with those expressed by mouse eTACs; human $AIRE^+$ cells failed to stain for CD45, and also did not appear to express CD86. Also, while no direct data on tolerance induction by human peripheral $AIRE^+$ cells is available, the authors did show that $AIRE^+$ cells may be inherently tolerogenic through their synthesis of anti-inflammatory IL-10 and through the transcription of tissue-specific antigens such as insulin. Human $AIRE^+$ cells stained positive for CD11c, a DC marker, and CD83, a marker for activation of dendritic and other cell types. In contrast, immunofluorescent analyses of Aire and Aire-driven GFP in mice have shown that mouse eTACs appear to lack CD11c expression [68]. More investigation of peripheral human $AIRE^+$ cells and their murine counterparts is warranted to clarify this discrepancy.

**Peripheral *Aire* expression may complement the function of Aire in the thymus**

In addition to determining the potential ability of eTACs to interact with cognate T cells, an analysis of the role of Aire itself in directing expression of tissue specific antigens in eTACs was conducted by sorting out eTAC-enriched fractions from *Aire* wild-type and knockout mice, and comparing their transcripts by microarray analyses [68]. Tissue-specific antigens were identified by being previously detected in 5 or fewer tissues analyzed during a whole organism genetic screen. The results were compared
with those obtained earlier for mTECs, and it was found that there were roughly 160 genes differentially regulated by Aire, that these targets were enriched for TSAs, and that the identified targets were distinct from those which were turned on in the thymus by Aire. A few of the most strongly Aire-regulated TSAs were confirmed to be differentially expressed between Aire wild-type and knockout eTACs using qPCR. This novel set of targets suggests that peripheral Aire may complement Aire’s role in the thymus, rather than simply reinforcing deletion to the same TSAs that are already expressed in the thymus.

The ability of eTACs to induce deletional tolerance among cognate T cells and their expression of putative tissue-specific antigens imply that they may have a physiological role in maintaining peripheral tolerance, although the experiments required to formally make that determination have yet to be conducted. While the surface marker profile of eTACs suggests that their ability to induce tolerance may be due to the lack of CD80 and CD86 on their surface, this question also warrants further investigation. Also, another outstanding question is how inflammation would affect the tolerogenic properties of eTACs, and how the cells themselves would respond to inflammatory stimuli in terms of homeostatic expansion or contraction. DCs are known to upregulate CD80 and CD86 in response to inflammatory stimuli, and it is worthwhile to determine whether the lack of CD80 and CD86 on eTACs is an inherent property, or whether they too upregulate these molecules in response to inflammation [90]. To address the second question, experiments conducted to identify eTACs in mice on the NOD or C57BL/6 background have not found significant differences in eTAC representation, suggesting that eTACs as
a whole are not overly sensitive to the relative differences in systemic inflammation between these strains.

While signals regulating peripheral Aire are unknown, recent data has suggested temporal regulation of Aire. Poliani et al. found that AIRE seemed to be absent from fetal lymph nodes, and was only present in postnatal tissues [89]. The finding that peripheral AIRE expression does not appear in humans until after birth contrasts with the appearance of Aire-expressing cells within the fetal thymus. Moreover, Poliani et al. also described an increase in AIRE\(^+\) peripheral cells throughout middle age, although the finding did not reach statistical significance. Likewise, preliminary data also suggests that eTACs increase in absolute number with the age of mice (Metzger et al., unpublished data). In contrast, the prevention of autoimmunity by Aire expression in the thymus seems to be most important during a perinatal window leading up to the age of weaning, or during conditions in which perinatal-like settings are experimentally induced through ablation of peripheral lymphocytes [31]. Therefore, peripheral Aire may represent a distinct method of establishing tolerance that becomes more important in aging as the burden of tolerance shifts from the thymus to the periphery (Figure 1.2).

**Aire-independent peripheral tissue-specific antigens may also contribute to peripheral tolerance**

In addition to the presence of Aire-driven peripheral TSAs as a possible mechanism of peripheral tolerance, other Aire-independent antigens with tolerogenic properties have been identified. Lee *et al.* found an unexpected activation of their
Figure 1.2. Central and peripheral Aire expression promotes tolerance through complementary mechanisms. Central Aire expression is most important during a perinatal window, and promotes tolerance through negative selection against tissue-specific antigens within the thymus. Peripheral Aire expression is likely to promote tolerance throughout life, and may induce deletional tolerance, Foxp3+ regulatory T cells, or anergy through the expression of a distinct set of peripheral tissue-specific antigens. The lack of CD80 and CD86 on peripheral eTACs is likely to be an important component of their tolerance induction.
Figure 1.2

**Aire+ mTEC**
Most important in *perinatal window*

Mechanism of *Central Tolerance*
- Negative selection
- Provides ligands
- Alter APC properties

**eTAC**
More important in *adulthood* (?)

Mechanism of *Peripheral Tolerance*
- Deletional tolerance
- Anergy (?)
- Adaptive T reg induction (?)

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**Surface Markers**

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iFABP-OVA transgene in all peripheral lymph nodes investigated [91]. Adoptively transferred cognate OT-I T cells experienced deletional tolerance in the lymph nodes, but not spleens, of iFABP-OVA mice, and failed to cause inflammatory lesions in the intestine, the standard site of iFABP transcription. This was likely to due deletional tolerance, as initial proliferation of transferred OT-I T cells gave way to a later contraction among the same population. Further experiments were done to characterize the lymph node cells responsible for transcribing the iFABP driven transgene, and it iFABP-OVA was found to be activated specifically in CD45−, radioresistant stromal cells in the lymph nodes, but not spleen. Furthermore, activation did not occur among CD11c+ cells, consistent with the lack of OVA transcription among cells of the hematopoietic lineage. Transcripts of other tissue-specific antigens were found among the lymph node stroma, including GAD67, and Aire transcript was also detected among this population, suggesting that peripheral Aire could be playing a role in driving these antigens. In fact, UEA-1 staining, which marks a subset of mTEC in the thymus including those that express Aire, was found among the lymph node stroma, along with the fibroblastic reticular cell marker gp38. These cells were found to preferentially interact with cognate OT-I T cells following their transfer when the stroma expressed cognate antigen.

While the work by Gardner et al. showed that Aire-expressing cells are gp38−, this finding was confirmed by later work using the iFABP-OVA mouse. These later experiments found that OVA was most highly transcribed by gp38+ CD31− fibroblastic reticular cells (FRC), and not by the gp38− CD31− fraction of lymph node stroma, where Aire transcript was most enriched [92]. Another piece of information demonstrating that eTACs and the iFABP-transcribing cells are distinct entities is the fact that Aire
expression is detected at roughly equivalent levels in the both the lymph nodes and spleen, but iFABP activation appears to occur exclusively in lymph nodes, and not in the spleen. Fletcher et al. also found that the FRC fraction of the lymph node was able to stimulate OT-I T cell proliferation \textit{ex vivo} when it contained the iFABP-OVA transgene, and furthermore, that this proliferation was subject to regulation by TLR3 signaling. Treatment of transgenic mice with a TLR3 ligand prior to analysis of their FRCs resulted in decreased expression of OVA and subsequently milder activation of cognate OT-1 T cells. Interestingly, no particular lymph node stromal fraction seemed to be uniquely responsible for the presence of the analyzed antigens. While iFABP-OVA activation occurs in cells that are distinct from the eTACs described by Gardner et al., these independent systems suggest that tissue-specific antigen transcription in the periphery capable of inducing clonal deletion may be an underappreciated phenomenon.

Tyrosinase is another antigen found to be naturally expressed in lymph nodes and, similarly to iFABP-OVA, can trigger deletional tolerance among cognate CD8$^+$ T cells [93]. Furthermore, tyrosinase presentation by stromal elements within the lymph nodes was shown to be sufficient to mediate this effect. Two groups found that gp38$^+$ CD31$^+$ lymphatic endothelial vessels were uniquely responsible for transcribing the tyrosinase antigen, at least in comparison with FRCs, blood endothelium, and the gp38$^-$CD31$^-$ quadrant of the lymph node stromal fraction, in which Aire expression has been detected [92, 94]. Therefore, the findings of the tyrosinase system complement those of the iFABP-OVA system; Aire-independent stromal cells can transcribe antigens capable of mediating deletional tolerance, at least through an MHC class I pathway. Further work on peripheral tissue specific antigens is warranted to determine whether these antigens
are in fact representative of a larger transcriptional profile within the lymph node with the function of inactivating cognate T cells, or whether these represent proteins whose transcription within lymph nodes serves a previously unappreciated function related to the function of the antigen itself. The applicability of these findings to MHC class II antigens should also be investigated, as it is unclear whether peripheral tissue-specific antigen transcription contributes to tolerance among CD4+ T cells.

Finally, the possibility that peripheral antigen induction may occur through a transcriptional activator other than Aire must be considered. In fact, evidence was recently put forth that Deaf1, a structural cousin of Aire through their shared presence of a SAND domain, may contribute to peripheral tissue-specific antigen activation in lymph nodes [95]. Transcripts of genes representing autoantigens in type I diabetes such as insulin were detected in the pancreatic lymph node of NOD mice, and their expression level was found to fluctuate in parallel with those of Deaf1. Furthermore, a comparison of Deaf1 knockout to wild-type mice by microarray revealed that, while Deaf1 deficiency does not affect peripheral Aire transcription, it does appear to affect transcription of tissue-specific antigens, and Deaf1 knockout mice develop eye-reactive autoantibodies on the BALB/c background. Also, the authors found a splice variant of Deaf1 that seemed to be particularly enriched among NOD mice, and suggested that the presence of this non-functional isoform, through sequestration of the standard Deaf1 isoform in the cytoplasm, could contribute to a reduction in Deaf1-controlled antigen expression. As a result of reduced peripheral tolerance induction to Deaf1-controlled antigens, the development of type I diabetes in the NOD mouse may be favored. Furthermore, the human equivalent of this variant Deaf1 isoform was shown to be 20-fold higher in
pancreatic lymph node sections from type I diabetics in comparison with control tissues, suggesting a possible maintenance of tolerance in humans by the standard DEAF1 isoform. An independent investigation found that Deaf1 was expressed in all lymph node stromal subsets investigated, and was most highly expressed among FRCs (90), suggesting that this putative transcriptional activator might represent another set of tissue-specific antigens. In tandem with Aire-directed antigens in eTACs and the class I antigens tyrosinase and iFABP in lymphatic endothelium and FRCs, respectively, DEAF1 may promote physiologically-relevant peripheral TSA expression.

**Summary**

Since the initial identification of the AIRE gene in 1997, significant progress has been made in understanding its critical contribution to processes of central tolerance. However, future work is required to expand our knowledge of Aire and its function, and some critical questions are addressed in the following chapters. The developmental pathway and turnover kinetics of Aire+ mTECs in the thymus have been incompletely understood, and experiments describing the recovery potential of Aire+ mTECs following ablation appear in Chapter II. Furthermore, previous reports have been conflicted in their description of whether or not Aire+ mTECs have further developmental potential, and new data resolving this issue also appears in Chapter II. Also, while much work has been done to describe the role of Aire in promoting central tolerance, its contribution to peripheral tolerance necessitates further investigation. Specifically, the identity of peripheral Aire-expressing cells has been unclear, and this
question will be further explored in Chapter III. Additionally, eTACs have been shown
to be capable of interacting and deleting CD8+ T cells in an antigen-specific manner, but
their ability to interact with CD4+ T cells has not been previously reported. This
question is addressed in Chapter IV, alongside an assessment of mechanisms contributing
to peripheral tolerance induction by Aire. Finally, the findings of this work along with a
discussion of their significance and suggestions for future areas of investigation are
presented in Chapter V.
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Chapter II

Lineage tracing and cell ablation identifies a post-Aire expressing thymic epithelial cell population
Introduction

Central tolerance in the thymus plays a critical role in preventing T cell reactivity to self and the prevention of autoimmunity [1]. Medullary thymic epithelial cells (mTECs) are a specialized antigen presenting cell type for guiding central tolerance, which they enforce through their expression of a wide array of tissue-specific self-antigens (TSAs) [2, 3]. TSA expression depends in part on Aire [4], which was originally identified as the defective gene in the monogenic, multi-organ autoimmune syndrome Autoimmune Polyglandular Syndrome Type 1 (APS1) [5-7]. While the molecular mechanisms by which Aire enables TSA expression in mTECs have yet to be fully elucidated, many individual Aire-dependent TSAs appear to have unique roles for enforcing proper central tolerance [8-11]. Reports following the initial association of Aire and TSA transcription found that transgenic Aire-dependent antigen expression enforced central tolerance primarily through negative selection [12, 13], and more recent work has found that endogenous Aire-dependent antigens can also mediate efficient negative selection of autoreactive epitope-specific T cell clones in a polyclonal setting [8, 14].

Following its initial identification, the expression pattern of Aire in the postnatal state has been found to be predominantly restricted to a subset of mTECs [2, 15-17], consistent with its critical role in central tolerance induction. The Aire+ mTEC subset uniformly exhibits high MHC Class II and CD80 expression [17, 18] and is thought to be most important for enforcing negative selection of autoreactive thymocytes, while Aire-negative mTEC subsets can express both high and low levels of these markers. Given the observations that MHC II<sup>lo</sup> mTECs precede MHC II<sup>hi</sup> mTECs during ontogeny[18, 19] and can give rise to MHC II<sup>hi</sup> mTECs in <em>in vitro</em> cultures [17, 20], MHC II<sup>lo</sup> mTECs
appear to represent a precursor of Aire+ mTECs. In line with this precursor-product relationship, many Aire-negative mTECs are actively dividing in a steady-state adult thymus, and presumably replace non-dividing Aire+ mTECs, which despite their lack of division undergo a substantial amount of turnover and replacement [17, 18]. However, this precursor-product relationship is an area of debate, as other evidence has suggested that Aire may mark an mTEC subset with further differentiation potential [21-23].

A number of signaling components converging on NF-κB activation have been demonstrated to play an important role in the development of mTECs, particularly the TNF receptor family members RANK and CD40 which signal through TRAF6 [20, 24-27]. Crosstalk between developing stromal cells and lymphoid cells bearing ligands for these receptors is required for proper mTEC differentiation, and non-conventional lymphoid cells appear to be the major source of these inductive cues during early stages of development [20, 28]. Additional signals, including ligands operating through the lymphotoxin β receptor, also contribute to proper mTEC development and homeostasis [29, 30]. Despite the clear requirement for these pathways in development, their role in the regulation of the adult mTEC compartment, which undergoes both homeostatic turnover and phases of involution and recovery following infection [31], remains undefined.

Here, we utilized genetic ablation and fate-mapping techniques to examine the role of Aire in the development and maintenance of the mTEC compartment at baseline and in response to dynamic changes. In order to allow controlled ablation of Aire-expressing cells in vivo, we developed an Aire-regulated bacterial artificial chromosome
transgenic mouse line that drives the expression of the high affinity diphtheria toxin receptor (DTR). Treatment of Aire-DTR transgenic mice with diphtheria toxin (DT) demonstrated that Aire-expressing mTECs are efficiently ablated, but long term DT treatment of Aire-DTR mice resulted in near complete ablation of the entire mTEC compartment and loss of immune tolerance. Detailed fate mapping experiments with a novel inducible Aire-Cre mouse strain revealed a surprisingly large and unique population of post-Aire expressing mTECs. These post-Aire mTECs maintained Aire-dependent TSA expression but reduced their expression of mTEC maturation markers and also became more centrally located within the medulla. Finally, DT-mediated mTEC ablation revealed a remarkable regenerative potential of mTECs to repopulate the thymus in a RANK-dependent manner.

Results

An Aire-DTR mouse allows targeted ablation of Aire+ mTECs

The importance of Aire in mediating selection events led us to create a transgenic mouse expressing the human diphtheria toxin receptor (also known as heparin-binding EGF-like growth factor) under the transcriptional control of Aire (Aire-DTR), in order to allow temporal control of Aire+ mTEC apoptosis following diphtheria toxin (DT) administration [32-35]. A previously characterized DTR-GFP construct [32, 34] was placed under control of the Aire promoter using a bacterial artificial chromosome [36] (Figure 2.1A). In Aire-DTR mice, DTR-GFP was expressed specifically by mTECs.
Figure 2.1. The *Aire-DTR* mouse facilitates efficient ablation of Aire+ mTECs. (A) Schematic illustration of the *Aire-DTR* transgene targeting expression of a DTR-GFP fusion protein to a 175 kb BAC containing the murine Aire locus. (B) Representative immunofluorescent staining of thymic sections from *Aire-DTR* mice stained for GFP (green) and Aire (red), with the DT treated recipient receiving a single dose of 50 ng/g DT 24 hours prior to analysis. Scale bars = 25 µm. (C) Comparison of percent GFP+ mTEC survival after two daily injections of the indicated dose of DT relative to an untreated *Aire-DTR* control, as assessed by flow cytometric detection of DAPI- CD45- MHC II+ GFP+ events. (E) Representative flow cytometric analysis of MHC class II and GFP expression in mTECs from the indicated mice, given one dose of 50 ng/g DT 24 hours prior to analysis. Plots are pre-gated on FSC/SSC, DAPI-, CD45-, EpCAM+, Ly51- events from stromal-enriched fractions. (E) Quantification of (D), showing total events per thymus of the indicated mTEC subsets, from two independent cohorts of mice, n=4.
Figure 2.1

A

B

C

D

E

Figure 2.1

A

B

C

D

E
within the thymus, and co-localized with Aire by immunofluorescence (Figure 2.1B). An initial assessment of DT-mediated ablation showed complete loss of Aire-expressing cells following a single injection of DT (Figure 2.1B), and a titration of DT dosing revealed a dose-response curve that was similar to that observed in other DTR ablation systems [32, 34] (Figure 2.1C). Flow cytometric analysis confirmed that GFP expression was specific to the MHC II hi mTEC subset, and that GFP+ mTECs were efficiently ablated with a single dose of DT (Figure 2.1D). We also observed some loss of GFP- mTECs with DT treatment, although the weakness of signal from the DTR fusion protein, as seen in other DTR systems [32, 37], underreported Aire expression to some extent (data not shown). Importantly, the MHC II lo fraction of mTECs remained intact after a single DT treatment. Outside of the thymus, a rare population of extrathymic Aire-expressing cells (eTACs) are present in the secondary lymphoid organs [36] and we also observed GFP expression and susceptibility to DT ablation by eTACs in Aire-DTR mice (data not shown). For clarity, we chose to focus our initial experimental efforts on the mTEC compartment in this study.

Extended Aire+ mTEC ablation depletes all mTEC subsets

We next sought to determine the effects of continuous Aire+ mTEC ablation on the architecture and cellularity of the medullary thymic compartment. We administered DT every 48 hours to a cohort of Aire-DTR mice, analyzed GFP+ mTEC ablation at various time-points after the beginning of treatment, and found that GFP+ mTECs were still completely ablated after five rounds of DT treatment. However, we observed recovery of GFP+ mTECs with DT treatment beyond this time-point (data not shown),
presumably due to antibody-mediated neutralization of the toxin, as anti-DT antibodies have been previously reported [38]. Thus, we chose to use a 9-day time-course of DT ablation, with 5 consecutive DT treatments, to examine the effects of repeated Aire+ mTEC ablation on the thymus. Surprisingly, we found that repeated ablation of Aire+ mTECs led to a substantial loss of all mTEC subsets, as assessed by cytokeratin 5 immunostaining (Figure 2.2A), with depletion of both GFP- MHC II<sup>hi</sup> mTECs as well as MHC II<sup>lo</sup> mTECs (Figure 2.2B, 2.2C). Interestingly, despite the absence of mTECs from these mice, CD4+ thymocytes and CD11c+ DCs were still observed throughout the medulla (Figure 2.2D). Total cell counts between ablated and control mice were similar, indicating that ablation in the Aire-DTR mouse was specific to the mTEC compartment and did not lead to a dramatic loss of developing T cells (Figure 2.2E).

**mTEC ablation leads to impaired CD4+ T cell selection**

To examine the effects of mTEC ablation on ongoing thymic selection in a polyclonal setting, we again continuously administered DT to a group of age-matched female mice. Interestingly, we observed a substantial increase in the proportion of CD4 single-positive (SP) thymocytes within mTEC-ablated thymi (Figure 2.3A), consistent with defective negative selection of a subset of the developing T cell pool in the absence of the mTEC compartment. Furthermore, we observed that the proportion of developing Foxp3+ regulatory T cells (Treg) among CD4 SP cells in the thymus was decreased (Figure 2.3B), although this was partially counterbalanced by the overrepresentation of total CD4 SP events in mTEC-ablated mice.
**Figure 2.2. Extended Aire+ mTEC ablation depletes all mTEC subsets.** (A) Representative immunofluorescent staining of thymi from the indicated mice stained for cytokeratin 5 (K5, green) and cytokeratin 8 (K8, red). DT treatments of 50 ng/g were given i.p. either 24 hours prior to analysis, or every 48 hours until analysis at day 9, as indicated. Broken lines indicate the cortico-medullary junction. Scale bars = 400 µm. (B) Representative flow cytometric analysis of MHC class II and GFP expression in mTECs from same conditions in (A). Plots are pre-gated on FSC/SSC, DAPI-, CD45-, EpCAM+, Ly51- events from stromal-enriched fractions. (C) Quantification of (B), showing total events per thymi of the Aire-DTR mice, from two independent cohorts of mice, n=4. (D) Representative immunofluorescence images of CD11c (green) and CD4 (red) staining of thymic sections from wild-type and Aire-DTR mice given repeated DT injections. Broken lines indicate the cortico-medullary junction. Scale bars = 400 µm. (E) Total thymic cellularity from littermate wild-type and Aire-DTR mice after receiving repeated DT treatment.
Figure 2.2
Figure 2.3. mTEC ablation leads to impaired CD4⁺ T cell selection and autoimmunity. (A) Representative flow cytometric plots of CD4 and CD8 staining and percents of CD4 SP events of FSC/SSC, DAPI- developing thymocytes in female mice of the indicated genotype at the end of repeated DT treatment. Data are representative of three independent experiments. (B) Representative histograms of Foxp3 staining among CD4 SP fractions shown in (A) for either wild-type (blue) or Aire-DTR (red) mice. (C) Representative flow cytometric plots of CD4 and CD8 staining and percents of CD4 SP events of FSC/SSC, DAPI- developing thymocytes in mice of the indicated genotype at the end of repeated DT treatment. Mean +/- SD CD4 SP percent is shown for 3-4 mice per group from two independent experiments. (D) Representative histograms of Foxp3 staining among CD4 SP events gated in (C). (E) Plot of mean weights over time of a cohort of 5-6 mice of each indicated genotype. Arrows at 7 and 21 days indicate the first and last DT injections, with 25 ng/g DT given i.p. every 48 hours during this window. (F) Representative flow cytometric plot of CD44 and CD62L staining of CD4⁺ splenocytes analyzed at 14 weeks of age. (G) Quantification of (F) for the cohort of mice in (E), left, and quantification of percent of Foxp3⁺ events among CD4⁺ splenocytes for the same cohort of mice. (H) Representative histological analyses of lacrimal glands from mice in (E) stained with hematoxylin and eosin (left) and infiltrate scores for the same cohort of mice (right). Scale bars = 100 µm.
Figure 2.3
We next turned to the RIP-mOVA model of neo-self-antigen expression within Aire+ mTECs, in which the rat insulin promoter drives expression of the model antigen ovalbumin (OVA) in a membrane-bound form [39] and examined how mTEC ablation affected negative selection of the OVA-specific OT-II CD4+ TCR transgenic line [13, 40]. We analyzed thymocytes from OT-II, OT-II RIP-mOVA and OT-II RIP-mOVA Aire-DTR mice that had received repeated DT treatments, and found that OT-II RIP-mOVA mice had dramatically reduced CD4 SP events, as reported previously [13]. In contrast, mTEC ablation in OT-II RIP-mOVA Aire-DTR mice completely removed OT-II mediated negative selection, and CD4 SP T cell development in OT-II and OT-II RIP-mOVA Aire-DTR mice was indistinguishable (Figure 2.3C). Furthermore, the development of Tregs observed in the presence of thymic OVA was also lost with mTEC ablation and the removal of cognate antigen from the thymus (Figure 2.3D). Finally, antibodies specific for the alpha and beta chains of the OT-II TCR confirmed that, while negative selection in RIP-mOVA mice led to a lower frequency and intensity of the OT-II TCR, OT-II T cells with normal antigen affinity developed in the absence of mTECs (Figure S2.1). Taken together, these results demonstrate that transgenic ablation of mTECs in the adult thymus leads to a loss of proper selection in both polyclonal and transgenic settings.

We next returned to a polyclonal setting to look for functional defects in tolerance resulting from mTEC ablation. Initially, we began by treating mice at birth, as this appears to be an important window for central tolerance induction to Aire-dependent antigens [41]. While others have reported a lack of non-specific DT toxicity in newborn
Figure S2.1. Negatively selected OT-II CD4+ T cells undergo TCR rearrangement. Representative flow cytometric plots of Vα2 and Vβ5 TCR signal among CD4 SP events gated in Figure 2.3C.
Figure S2.1

The figure shows a dot plot analysis comparing different conditions labeled OT-II, OT-II RIP-mOVA, and OT-II RIP-mOVA Aire-DTR. The x-axis represents the % of OT-II TCR+ cells, while the y-axis indicates the Vb5 and Va2 expression levels. The graph includes statistical significance measures with a p-value of 0.0033.
mice [34], treatment of mice with a maximally effective dose of DT from birth was lethal in our mouse colony (data not shown). We next treated mice beginning at one week of age, and found that weight gain by the treated mice slowed towards the end of the treatment regimen (Figure 2.3E), but that the mice survived and gained weight normally after weaning. We tracked the mice through 14 weeks of age, and while we did not observe signs of autoimmunity through weight loss, we did find a substantial increase in the proportion of CD44+ CD62L- memory/effector T cells in the periphery at 14 weeks of age (Figure 2.3F), suggesting an increase in the leaking of autoreactive T cells from the thymus following mTEC ablation. Unlike the decreased proportions of thymic Tregs we observed immediately following mTEC ablation, we found increased percentages of peripheral Tregs in mice that had experienced mTEC ablation before weaning (Figure 2.3G). Lastly, we observed a significant increase in tissue infiltrates of lacrimal glands (Figure 2.3H), demonstrating that mTEC ablation in the postnatal thymus leads to a functional break in tolerance.

**Lineage tracing identifies a post-Aire, MHC II<sup>lo</sup> stage of mTEC development**

Our observation that repeated DT-mediated ablation of Aire+ mTECs led to ablation of mTECs outside of this pool led us to address whether or not there might be a post-Aire stage of mTEC development. Towards this end, we created an inducible Aire-Cre line in which tamoxifen treatment would allow nuclear localization and loxP-directed excision in Aire-expressing cells (Figure 2.4A). Because a wave of transient Aire
Figure 2.4. The Aire-Cre mouse allows inducible labeling of Aire-expressing mTECs. 

(A) Schematic illustration of the Aire-Cre transgene targeting expression of an ERT2-Cre-ERT2 fusion protein to Aire+ cells through a 175 kb BAC containing the Aire locus (top). When crossed with the Ai14 Rosa26 RFP mice (bottom), tamoxifen-induced Cre activity excises the stop codon and allows constitutive expression of RFP (TdToma) in labeled cells. (B) Flow cytometric analysis of thymic stromal fractions from Aire-Cre x Ai14 mice given 7 mg tamoxifen or corn oil at day 0 and day 3 and analyzed on day 5. Plots show EpCAM and RFP signal on FSC/SSC, DAPI-, CD45- events. (C) Representative immunofluorescent staining of Aire and RFP from thymic sections of the same mice as (B), with mock corn oil or tamoxifen injections indicated. Scale bars = 50 µm, left and middle, 20 µm, right.
**Figure 2.4**

**A**

- Aire Ex1
- B-globin splice
- SV40 pA
- Aire Ex3

- 90kb
- 85kb

**B**

- Mock
- Tamoxifen

- EdCAM
- RFP

**C**

- Mock
- Tamoxifen
- Tamoxifen

- Aire RFP
expression occurs during embryogenesis [23], we used a previously described approach of dual flanking of the Cre recombinase with tamoxifen receptor cassettes to allow strict temporal control of Cre activity [42]. We then crossed these mice with a mouse strain harboring Cre-inducible TdTomato (RFP) under control of the ubiquitous Rosa26 promoter to generate mice where Aire-expressing cells would be irreversibly labeled through constitutive RFP expression following tamoxifen treatment. To validate the function of our Aire lineage-tracing mice, we administered two repeated doses of tamoxifen and observed strong RFP labeling of a subset of EpCAM+ mTECs (Figure 2.4B). RFP-labeled cells had a high degree of co-staining with nuclear Aire protein as expected (Figure 2.4C) and, importantly, we saw no evidence of RFP labeling in the absence of tamoxifen treatment (Figure 2.4B, 2.4C), indicating the absence of background Cre activity.

We next addressed the developmental fate of Aire+ mTECs by delivering a single dose of tamoxifen and analyzing the characteristics of RFP-labeled mTECs over time. We observed a high degree of co-expression of RFP and Aire-driven GFP at only 24 hours post-tamoxifen treatment [36] (Figure 2.5A), demonstrating highly specific labeling of Aire+ mTECs at day 0. However, when we examined Aire-GFP expression by RFP+ mTECs a week or more after a single dose of tamoxifen treatment, we were surprised to find that many labeled mTECs were no longer actively expressing Aire, and that roughly half of all labeled mTECs progressed to this post-Aire stage within 10 days (Figure 2.5B). Importantly, we found that the overall decay in labeled Aire+ GFP+ cells
showed a half-life of about 7 days, consistent with previous reports [17], but when post-Aire cells were taken into account, labeled cells remained present in relatively stable

**Figure 2.5. Post-Aire mTECs downregulate MHC Class II.** (A) Representative flow cytometric analyses of *Aire-Cre x Ail4(Rosa26-RFP) x Aire-GFP (Adig Aire-reporter)* mice treated with 2 mg tamoxifen at d0 and analyzed at the indicated timepoints. Plots are pre-gated on DAPI-, CD11c-, CD45-, EpCAM+ events, and representative of two independent experiments, n=4. (B) Plots of mice analyzed in the same manner as (A), showing mean and SD over time of GFP+ RFP+ events (black), GFP- RFP+ events (red), or both GFP- and GFP+ RFP+ events (blue). (C) Representative histograms of MHC Class II expression by GFP and RFP subsets gated in (A) showing GFP- RFP- (black), GFP+ RFP+ (blue), or GFP- RFP+ (red). (D) Representative immunofluorescent analyses of thymi from mice shown in (A) with overlays of GFP (green), RFP (red) and/or Aire (blue) as indicated. Scale bar = 50 µm. (E) Schematic of sub-regions of the medulla for which densities were calculated in the following displays. Concentric 50 um regions starting from the cortico-medullary boundary were defined, with the outer region most proximal to the boundary, and the density of mTEC subsets within each region was determined. (F) Calculation of GFP+ mTEC densities (cells per 100 um²) among the entire medullary region (all) and within the three concentric regions depicted in (E). Data are from 21 medullary regions imaged from four separate thymi of *Aire-Cre x Ail4(Rosa26-RFP) x Aire-GFP (Adig Aire-reporter)* mice 21 days after tamoxifen treatment. (G) Calculation of RFP+ mTEC densities as in (F). (H) Comparison of the ratio of GFP and RFP densities across the outer and mid regions showed statistical significance using a paired t-test.
Figure 2.5
proportions within the first week of labeling (Figure 2.5B). Nevertheless, at one week after labeling, post-Aire mTECs decayed at the previously reported rate, suggesting a lack of further differentiation or expansion capacity among this population. We also examined MHC Class II expression on post-Aire mTECs and found that, in addition to losing expression of Aire-GFP signal and Aire protein (Figure S2.2A), a substantial fraction of post-Aire mTECs could also revert to an MHC IIlo phenotype (Figure 2.5C). By immunofluorescent staining, we again found that while Aire-GFP, RFP, and nuclear Aire protein co-localize shortly after tamoxifen treatment, by day 7 post-tamoxifen treatment, many post-Aire cells express only RFP (Figure 2.5D). Post-Aire cells at 21 days after labeling continued to be localized to the medulla, and while Aire and Aire-GFP signals tended to outline the edge of the medullary region, as shown previously [16, 22], post-Aire RFP cells appeared to be preferentially localized towards the center of the medulla by 2D and 3D imaging (Figure S2.2B, S2.2C). We further investigated the differential localization of Aire+ and post-Aire mTECs 21 days after labeling and found that while Aire+ mTECs were preferentially localized to the outer portion of the medulla, as described previously [22, 43], post-Aire mTEC distribution was skewed towards the center of the medulla (Figure 2.5E-H).

Given the phenotypic similarity between MHC IIlo post-Aire mTECs and the potential mTEC progenitor population, we sought to directly assess the potential of post-Aire mTECs to contribute to mTEC cellularity followed DT-mediated mTEC ablation. We labeled Aire+ mTECs through tamoxifen treatment, waited for a fraction of labeled mTECs to turn off Aire expression, and then ablated remaining Aire+ mTECs through the Aire-DTR transgene (Figure S2.2D). In line with the homeostatic decay of post-Aire
Figure S2.2. Post-Aire mTECs have a distinct localization within the medulla and do not contain mTEC progenitor capability. (A) Representative flow cytometric analysis of an *Adig x Aire-Cre x Ai14* (Rosa26-RFP) mouse treated with tamoxifen 7 days prior to analysis. mTECs were identified as CD11c- (not shown) and CD45- EpCAM+ (left). RFP was observed among a subset of mTECs (upper right), and MHCII and Aire protein expression is shown for this population (lower right). (B) Representative low power immunofluorescent image of GFP (green), RFP (red), and DAPI (blue) from an *Adig x Aire-Cre x Ai14* (Rosa26-RFP) mouse treated with tamoxifen 21 days prior to analysis. Note the enrichment of GFP- RFP+ cells in more central regions of the medulla. Scale bar = 400 µm. (C) Image represents 80 µm z-projections by multiphoton imaging of endogenous GFP (green) and RFP (red) in live thymic sections from an *Adig x Aire-Cre x Ai14* (Rosa26-RFP) mouse treated with tamoxifen 11 days prior to analysis. Scale bar = 30 µm. (D) Schematic of tamoxifen (2 mg oral gavage) and DT or PBS (50 ng/g i.v.) treatment of *Aire-Cre x Ai14* (Rosa26-RFP) *x Aire-DTR* mice. (E) Relative representation of RFP+ mTECs by FACS analysis in DT treated mice (white) standardized to RFP expression in PBS treated mice (grey) at each timepoint.
Figure S2.2

A

B

C

D

E

GFP / RFP / DAPI

Tamoxifen tx
DT or PBS tx
1d
5d
analysis

Aire-Cre
Rosa26-RFP
Aire-DTR

Percent of PBS-Tx

1d recovery
5d recovery

RFP+ mTECs

PBS

DT

1.47

14.3

26.2

37.6

35.9

0.28

0

50

100

150

GFP / RFP
mTECs, we observed that RFP-labeled mTECs were relatively scarce after recovery from a single DT ablation (Figure S2.2E), thus further supporting a linear relationship between Aire-negative mTEC precursors, Aire+ mTECs, and a final, distinct post-Aire state.

**Post-Aire mTECs lose maturation markers but maintain intermediate TSA expression**

Aire expression in mTECs is critical for the proper expression of TSAs within the thymus [4]. Thus, we addressed whether TSA expression occurred only in mTECs that were actively co-expressing Aire, or whether Aire might permit the further differentiation of mTECs into a post-Aire, TSA-expressing state [22, 44]. We identified post-Aire mTECs by sorting out MHC II$^{lo}$ RFP+ mTECs, which uniformly lack Aire protein (Figure 2.6A), 7 days after a single tamoxifen labeling and compared gene expression with other mTEC fractions. Importantly, expression of Aire and MHC II at the transcript level mirrored our observations at the protein level, and the expression patterns of the maturation markers CD80 and CD86 were also consistent with the loss of a mature phenotype by post-Aire mTECs (Figure 2.6B). We also found that involucrin (Ivl), a marker suggested to identify the last stages of mTEC development [22, 30], was markedly increased in the post-Aire lineage. Lastly, we addressed the relative levels TSA expression by post-Aire, MHC II$^{lo}$ mTECs and other mTEC subsets, and found that post-Aire mTECs continued to express TSAs at a significantly higher level than RFP-MHC II$^{lo}$ mTECs, which were enriched for pre-Aire mTECs (Figure 2.6C). Importantly,
Figure 2.6. Post-Aire mTECs lose maturation markers but maintain intermediate TSA expression. (A) Representative flow cytometric analysis of an Adig x Aire-Cre x AiI4 (Rosa26-RFP) mouse treated with tamoxifen 7 days prior to analysis. mTECs were identified as CD11c- (not shown) and CD45- EpCAM+ (left). A polychromatic plot shows the distribution of MHCII (green) and RFP (red) signal among TECs (middle), and Aire staining of the indicated quadrants (MHCII\(^{hi}\) RFP\(^{-}\), green; MHCII\(^{hi}\) RFP\(^{+}\), yellow; MHCII\(^{lo}\), RFP\(^{+}\), red) is shown to the right, demonstrating the lack of Aire expression among RFP+ MHCII\(^{lo}\) events. (B) Quantitative PCR analyses of indicated maturation targets (H2-Ab = MHC Class II, Ivl = Involucrin) on populations depicted in (A). Results are representative of 2 or 3 independent sorts. (C) Quantitative PCR analyses of indicated Aire-dependent TSA targets (left) and the Aire-independent TSA CRP (C-reactive protein) on populations depicted in (A). Results are representative of 2 or 3 independent sorts.
Figure 2.6

A

B

Lineage Markers

C

Tissue-Specific Antigens

74
post-Aire mTECs expressed TSAs at lower levels than the mature, Aire+ mTEC-containing subset, which suggests a direct role for Aire in facilitating TSA expression but not maintaining it. These results identify post-Aire MHC IIlo mTECs as a population with distinct TSA and antigen-presenting capabilities, which may indicate a unique role for this population in driving central tolerance.

**RANK signaling controls Aire+ mTEC homeostasis and regeneration**

While previous reports have noted a capacity for involution and regeneration by thymic epithelial cells in response to pharmaceutical agents [45], direct ablation and recovery of mTECs has not yet been addressed. We thus returned to a short-term targeted ablation of Aire+ mTECs to address the regulation of Aire+ mTEC development in the adult thymus. Following a single DT-mediated ablation of Aire+ mTECs (**Figure 2.7A**), we observed that a substantial number of Aire+ GFP+ mTECs were regenerated within three days (**Figure 2.7B**). Furthermore, FACS analysis of thymi at several stages of recovery revealed that mTEC subset ratios had returned to normal proportions within a week of ablation (**Figure 2.7C**). Prior studies have suggested that Aire+ mTECs develop from an immature MHC IIlo CD80- precursor population [17, 20], and in line with these observations, we addressed whether or not mTEC recovery might be impaired after a longer DT-mediated ablation regimen in which all subsets of mTEC are ablated. In line with these studies, we found that the additional loss of Aire-negative mTECs with repeated DT treatment led to an impaired recovery potential seven days after cessation of the repeated DT regimen (**Figure 2.7D-F**). Importantly, we observed that ablated
Figure 2.7. RANK signaling controls Aire+ mTEC development. (A) Schematic of single DT treatment (50 ng/g) of Aire-DTR mice and subsequent timepoints of analysis. (B) Representative immunofluorescent analysis of serial sections from mice after 1 or 3 days of recovery, with GFP (green) and Aire (red) above, or cytokeratin 5 (K5; green) and cytokeratin 8 (K8; red). Scale bars = 100 µm. (C) Quantification of flow cytometric analysis of thymi at the indicated timepoints of recovery from DT treatment, showing mean and SD of the percent of mTECs in either MHC II lo, MHC II lo GFP-, or MHC II lo GFP+ gates. n=4 per timepoint, pooled from two independent experiments. (D) Schematic of repeated DT treatment and recovery analysis at one or three weeks after the end of DT treatment. (E) Representative immunofluorescent staining of K5 (green), K8 (red), or Aire (blue) in thymic sections of the indicated genotype after one week of recovery from mTEC ablation. Scale bars = 400 um. (F) Quantification of flow cytometric analysis of thymi at the indicated timepoints of recovery from mTEC ablation, showing mean and SD of the total numbers of MHC II hi mTECs per thymus. (G) Schematic of DT treatment and i.p. injections of either anti-RANK-L or isotype control (upper left) followed by analysis after 5 days of recovery (upper left), and representative flow cytometric of MHC II and Aire staining of FSC/SSC, CD11c-, CD45-, EpCAM+ mTECs from mice with the indicated genotypes and treatments. n=4 per condition. (H) Representative histograms of Ki67 staining of populations in (G) for PBS (blue) or DT (red) treated, isotype treated DTR+ mice (top), and quantification of mean and SD of percent Ki67+ events among the indicated mTEC subsets from DTR+ mice receiving either PBS and isotype (blue), DT and isotype (red), or DT and anti-RANK-L (grey).
Figure 2.7
mTECs were ultimately able to recover to the same levels as their wild-type controls, suggesting that repeated DT treatment quantitatively but not qualitatively affects the recovery potential of mTECs.

RANK signaling through TRAF6 is an important mediator of Aire+ mTEC development [20, 24-26], but the importance of this pathway has been established primarily during thymic organogenesis. In order to determine the contribution of RANK signaling to the recovery of Aire+ mTECs in adult mice, we blocked RANK signaling with an anti-RANK ligand antibody during recovery from a single DT treatment (Figure 7G). Interestingly, we found that blockade of this pathway substantially reduced the proportion of Aire+ mTECs present in control mice after five days of RANK signaling blockade (Figure 2.7G), consistent with a high degree of constitutive Aire+ mTEC turnover [17] and a requirement for RANK signaling in this process. In the context of recovery from Aire+ mTEC ablation, we observed that isotype-treated mice recovered completely, and exhibited the previously reported phenomenon of Aire+ mTEC overshoot [45], which was more apparent than when indirectly assessing Aire expression through GFP (Figure 2.7C). Additionally, the effect of RANK signaling blockade was more dramatic in the context of recovery from DT-mediated Aire+ mTEC ablation, as this process was completely blocked in the absence of RANK signaling (Figure 2.7G). Finally, we assessed the proliferative response of mTEC subsets during mTEC recovery by Ki67 staining, and found that RANK signaling was required to allow enhanced proliferation of Aire-negative mTEC subsets during ablation recovery (Figure 2.7H). Taken together, these results identify a striking capacity for RANK-dependent Aire+ mTEC induction and repopulation of the adult thymus.
Discussion

Since the original identification of mTECs as the source of both Aire and TSA expression in the thymus [2, 15], there has been debate about the role of Aire and of epithelial cell maturation in driving TSA expression [4, 44], and the developmental relationships of Aire+ and Aire-negative mTECs has remained unclear. Here, using two separate genetic systems, we have provided new insights into the mTEC compartment and its maintenance in the adult thymus and have shown that: (1) repeated ablation of Aire+ mTECs leads to an unexpected loss of Aire-negative mTEC subsets; (2) following Aire induction among maturing mTECs, Aire is downregulated in a final post-Aire mTEC stage that retains TSA expression and acquires a distinct anatomical localization with the medulla; and (3) the recovery potential of Aire+ mTECs during adulthood depends on RANK signaling and correlates with the availability of precursor MHC IIlo cells.

Aire-negative mTECs are lost following repeated Aire+ mTEC ablation

In order to investigate the relationships of Aire+ and Aire-negative mTEC subsets, we created an Aire-DTR mouse in which Aire+ mTEC ablation could be temporally controlled. Surprisingly, we found that while a single DT treatment caused specific ablation of mature mTECs, repeated DT-mediated ablation led to a partial loss of the putative immature mTEC population as well. The loss of mTECs was not a result of general thymic atrophy, as total thymic cellularity remained unchanged with ablation of the mTEC compartment, and both thymocytes and DCs continued to fill the thymic...
medulla. In contrast to evidence that Aire+ mTECs represent a terminally differentiated population [17], these results suggested that Aire+ mTECs could contribute to the MHC II$^+$ pool of mTECs. In fact, recent experiments with constitutive Aire-Cre labeling have indicated that Aire+ mTECs may decrease their CD80 expression and adopt a more immature phenotype [23], although the specificity of initial labeling in this experimental approach was unclear.

Prior work has found that handoff of antigen from mTECs to DCs can be required to drive negative selection and central tolerance [46, 47]. Therefore, it was unclear whether mTEC ablation over the timespan of 9 days would be sufficient to remove functionally relevant mTEC-derived antigens from the thymus, or whether such antigens might persist and continue to be presented by DCs. To test the functional effects of mTEC ablation, we first examined polyclonal thymocytes, and found that CD4 SP thymocytes were over-represented in conditions of mTEC ablation. These results suggested that a substantial proportion of thymocytes, which would otherwise undergo deletion, were instead surviving in the absence of mTEC-derived antigen. The loss of negative selection to mTEC-derived antigen became much more striking when we performed similar experiments in the context of RIP-mOVA-mediated deletion of OT-II T cells. Importantly, this result determined that antigen handoff and persistence in DCs was not sufficient to mediate detectable negative selection during the duration of mTEC ablation that we induced.

Finally, we found that mTEC ablation in a polyclonal setting prior to weaning led to autoimmunity in adulthood. It should be noted that eTACs were also ablated in this experiment, which could have contributed to the observed autoimmunity, although a lack
of specific TSA expression in the thymus likely explains our observation of lacrimal gland autoimmunity [10]. Interestingly, the lacrimal gland infiltrates we observed have been one of the more penetrant phenotypes with a lack of proper Aire function [40, 48], and we expect that more extensive autoimmunity would have been observed if earlier DT administration was feasible [41]. Taken together, these results show that mTEC ablation in the Aire-DTR mouse leads to a loss of negative selection to mTEC-expressed antigens, and further highlights the critical role of mTECs in providing unique ligands for thymic negative selection.

Aire+ mTECs lose Aire and MHC II in a final post-Aire stage

By developing an inducible Aire-Cre mouse, we were able specifically label Aire-expressing cells with constitutive RFP expression and follow the developmental fate of these cells. Importantly, in contrast to previous reports with a germline Aire-Cre labeling system [23], our labeling was highly specific to cells actively expressing Aire within 24 hours of tamoxifen-induced labeling. Surprisingly, we observed that a substantial portion of Aire+ mTECs lost Aire expression within a week of labeling, and many of these post-Aire mTECs also went on to lose MHC class II expression and phenotypically resemble MHC IIlo immature mTECs. Over 10% of all mTECs adopted a post-Aire fate in our experiment, and this is an underestimate of the total proportion of post-Aire mTECs within the thymus, given our incomplete and temporally-restricted labeling of Aire+ mTECs. The lack of co-labeling of RFP and either Aire-GFP or Aire protein became striking by immunofluorescence 7 days after tamoxifen treatment, and we observed a
change in the distribution of post-Aire cells within the medulla after 21 days of labeling. The identification of a post-Aire mTEC developmental stage explains at least in part our observation that continued ablation of Aire+ mTECs leads to a loss outside of the Aire+ mTEC compartment. To address the contribution of post-Aire mTECs to thymic cellularity, we tracked the frequencies of labeled mTECs over time, and found that post-Aire mTECs were ultimately cleared from the thymus and replaced by cells without a prior history of Aire expression. We also addressed the potential of post-Aire mTECs to contribute to thymic cellularity during recovery from targeted Aire+ mTEC ablation, and again observed that post-Aire cells did not contain regenerative potential. Thus, our observation of a post-Aire mTEC state was consistent with our observations in the DT-mediated mTEC ablation and recovery, in that non-Aire+ mTECs could be ablated, or prevented from forming, through continued DT treatment, but the fundamental regenerative capacity of the medullary thymic epithelium was not dependent on Aire+ mTECs.

The loss of Aire by post-Aire mTECs is consistent with a model in which Aire drives further maturation of the mTEC subset [21]. However, we found that Aire-dependent TSA expression was highest in MHC II high cells, and decreased following transition of post-Aire mTECs into the MHC II low state. Thus, at least in terms of TSA expression, Aire seems to directly drive or stabilize expression of these transcripts [49], rather than facilitate transition into a specialized TSA-expressing post-Aire cell. In line with this, recent work has found that TSAs expressed endogenously in the pancreas do not require canonical pancreatic differentiation transcription factors for thymic expression [50]. Nevertheless, the persistence of TSA expression beyond the expression
of Aire itself suggests that post-Aire mTECs may have a role in inducing tolerance to TSAs, and could explain detection of TSAs among Aire-negative mTECs [51-53]. TSA persistence may be related to suggested mechanisms of Aire function in which Aire acts to open TSA loci through epigenetic modification rather than directly binding and driving transcription at target loci [49]. Interestingly, we found that while canonical mTEC maturation markers including CD80 and MHC class II were decreased in post-Aire mTECs, involucrin, a potential marker of a post-Aire state [22, 30], was in fact enriched in our post-Aire cells. Thus, the post-Aire mTEC developmental stage has a distinct transcriptional profile from the general pool of MHC IIlo mTECs, despite being indistinguishable by general maturation markers.

The lower levels of TSAs and antigen presentation capabilities in post-Aire mTECs suggests this developmental state may support a feature of central tolerance other than negative selection of autoreactive T cells. Instead, such post-Aire cells may provide architectural cues to the maintenance of the thymic medulla, and we have observed altered localization of post-Aire mTECs within the medulla. Indeed, the loss of the entire medullary compartment with extended ablation of Aire+ mTECs may have occurred not only due to the loss of both Aire+ and post-Aire mTECs, but also from a loss of important organizational cues provided by post-Aire mTECs. Proper architectural organization is likely required for appropriate regulatory T cell induction, as recent work has shown that altered orientation of medullary components in the absence of XCL1 chemokine signals leads to impaired regulatory T cell induction [54]. Furthermore, we speculate that post-Aire mTECs may also have a direct role in induction of regulatory T cells, consistent with the lack of regulatory T cells following extensive ablation of both
Aire+ and post-Aire mTEC in OT-II transgenic and polyclonal models. The reduced levels of both MHC II and antigen on post-Aire mTECs may promote regulatory T cell survival over deletion or neglect, and in line with a requirement for intermediate TCR signaling in regulatory T cell development, recent work has shown that the partial inhibition of MHC Class II expression by mTECs leads to enhanced selection of regulatory T cells [55].

**RANK signaling controls Aire+ mTEC homeostasis and regeneration**

Our investigation into the recovery potential of mTECs in the adult mouse revealed a remarkable capacity of Aire+ mTECs to be regenerated following diphtheria toxin-mediated ablation of this mTEC subset. The ability of the Aire+ mTEC pool to recover from targeted ablation was much more dramatic that both our own and prior estimates of the homeostatic turnover of this compartment [17, 18]. We found that the regenerative capacity of the Aire+ mTEC subset correlated with the presence of the MHC II lo subset, as MHC II hi mTECs remained underrepresented for at least one week following more thorough mTEC ablation. These results support a precursor-product relationship among at least a subset of mTECs within the MHC II lo pool and Aire+ mTECs.

We also found that recovery of the Aire+ mTEC subset following targeted ablation was critically dependent on signaling from RANK-L, as a blocking antibody to this ligand completely prevented the re-emergence of Aire+ mTECs after DT treatment.
Previously, it has been suggested that RANK signaling might be important predominantly during initial stages of mTEC development [27]. However, the data here clearly demonstrate a significant contribution of RANK signaling to the homeostatic turnover of the Aire+ mTEC subset in control, non-ablated adult mice, thus highlighting the importance of RANK signaling in the induction of Aire+ mTECs in adulthood. Interestingly, our analysis of mTEC proliferation during recovery from diphtheria toxin ablation suggested that RANK signaling might allow the maturation and induction of Aire expression by controlling proliferation of precursor populations, as mTEC proliferation across all subsets was suppressed to homeostatic levels in the absence of RANK signaling.

Taken together, these results demonstrate that the mTEC compartment is highly dynamic and provide evidence of rapid turnover and tight regulation of Aire expression. This coordinated expression and turnover may be critical for the maintenance of central tolerance. In this regard, the continued turnover and replacement of Aire mTECs may help ensure a broad display of TSAs to the developing T cell repertoire. Detailed analysis of TSA expression has demonstrated that this process is stochastic with a somewhat random distribution of TSAs in mTECs [52, 53]. Through rapid turnover and replacement of mTECs, random TSA expression in any single mTEC can still be anatomically and spatially spread out in the medullary compartment for the maintenance of tolerance to TSAs. Finally, our results make important modifications to the prevailing model of Aire+ mTECs representing a terminally-differentiated cell population by identifying a post-Aire stage in which both Aire and maturation markers are lost. The
existence of this post-Aire state suggests that future work addressing properties of mTEC subsets should seek to further identify pre- and post-Aire mTECs, and may help in more precisely identifying which mTEC subsets harbor the dramatic regenerative potential which we observed in our DT ablation system.

Materials and Methods

Mice. B6.OT-II mice (Jackson), B6.RIP-mOVA mice (kindly provided by M. Krummel), and B6/129.Ai14 (Rosa26-TdTomato, Jackson) mice were described previously [39, 56, 57]. All mice were housed in a specific-pathogen free facility at University of California, San Francisco (UCSF), and the UCSF Institutional Animal Care and Use Committee approved all experiments.

NOD.Aire-DTR mice. NOD.Aire-DTR mice were generated by standard cloning methods using a bacterial artificial chromosome (BAC) recombineering and transgenesis strategy. Briefly, the DTR-EGFP sequence was amplified from the pCD11c-DTR-EGFP vector using the primers 5’-TTAATTAGGGACCATGAAGCTGCCGTG-3’ and 5’-CGCGGCCGCTTTACTTGTA-3’. This PCR product was sub-cloned into pCR-Blunt II-TOPO vectors (Invitrogen), sequenced, then digested with PacI and NotI and inserted into pAireFNF, a BAC-targeting plasmid containing 5’ and 3’ homology arms to the mouse Aire gene. pAireFNF itself was generated from JDFNF, an Aire BAC-targeting expression construct previously described [36]. To generate pAireFNF, the multiple cloning site from the vector pLitmus 38i (New England Biolabs, Ipswich, Massachusetts) was amplified with the primers 5’-GGTTAATTAACTTCTGCAGGATATCTG-3'/5’-GGCCTGCAGGGCCTTGACTAGGGTCA-3’, subcloned into pCR-BluntII-TOPO, sequenced, and ligated by NotI partial/HindIII complete digest into JDFNF. This targeting construct, pAireFNF-DTR-EGFP, was then inserted via standard recombineering [58] into BAC clone 461e7 from the RPCI 23 Mouse BAC Library at the
Highly purified Aire-DTR BAC DNA was carefully prepared and injected into fertilized NOD oocytes by the UCSF Mouse Transgenic Core with the assistance of H. Lu and N. Killeen. NOD.Aire-DTR were backcrossed onto the C57Bl/6 strain, and B6.Aire-DTR mice were crossed with B6.RIP-mOVA and B6.OT-II mice after 4 generations of backcrossing to C57Bl/6 mice. B6.Aire-DTR mice were backcrossed 10 generations for RANK-L blocking experiments.

Aire-Cre mice. Aire-Cre mice were generated by standard cloning methods using a bacterial artificial chromosome (BAC) recombineering and transgenesis strategy. Because the Aire gene is expressed early in development[23], we chose to use a tamoxifen-inducible Cre cassette to prevent Cre-mediated excision early in development and utilized the previously described ERT2-Cre-ERT2 cassette[42]. Briefly, the ERT2-Cre-ERT2 expression cassette was excised from pCAG-ERT2-Cre-ERT2 (obtained from Dr. Connie Cepko, Harvard Medical School) by digestion with SmaI and Not I. This fragment was ligated into the pAireFNF targeting construct described above to generate pAire FNF-ERT2-Cre-ERT2. This targeting construct was then inserted via standard recombineering into BAC clone 461e7 from the RPCI 23 Mouse BAC library. Highly purified Aire-Cre BAC DNA was carefully prepared and injected into fertilized FVB oocytes by the UCSF Mouse Transgenic core with the assistance of H. Lu. Lineage tracing analyses were conducted on mice backcrossed at least 3 generations to C57Bl/6. Analyses on sorted cells were from Aire-Cre mice backcrossed at least 6 generation to C57Bl/6, and Aire-Cre x Aire-DTR mice were backcrossed at least 8 generations to C57Bl/6.

In vivo mouse treatments. Diphtheria toxin (DT, Sigma-Aldrich) was administered at a dose of 25-50 ng/g via i.p. injections for all experiments, with the exception of the RANK-L blocking experiment, in which DT was given via retro-orbital injections to mice anesthetized with a ketamine/xylazine mixture. RANK-L (clone IK22/5) or a Rat IgG2a isotype control (clone 2A3) were purchased from Bio-X-Cell and injected i.p. at 250 ug per dose as indicated. Tamoxifen (Sigma-Aldrich) was dissolved in corn oil
(Sigma-Aldrich), and 2 mg doses were administered by oral gavage unless otherwise indicated. All treatments were performed on mice near 6-8 weeks of age.

**Flow Cytometry.** Cells were isolated for analysis by mashing for lymphocyte analysis, followed by ACK lysis for spleen samples. TECs were isolated by mincing and collagenase/dispase digestion, as reported previously[36], followed by stromal cell enrichment on gradients of Percoll PLUS, purchased from GE Healthcare. Isolated cells were blocked with 2.4G2 antibody diluted 1:50 from 1 mg/mL stock, and stained with the indicated surface marker antibodies from BioLegend. For intracellular staining, anti-Aire-A647 and anti-Ki67-PE were purchased from eBioscience and BD Biosciences, respectively, and cells were permeabilized and stained with the eBioscience Foxp3 / transcription factor buffer set. Stained cells were analyzed on BD LSRRII and Fortessa cytometers, and cells were sorted on BD FACSARia III cytometers. Data was analyzed with BD FACS Diva and Flow Jo (Tree Star).

**Quantitative PCR.** Sorted cells were collected in a 50:50 mixture of DMEM and FBS, and RNA was extracted with a Qiagen RNeasy Micro kit, according to the manufacturer’s instructions. cDNA was synthesized with an Invitrogen Superscript III kit, and inventoried Applied Biosystem Taqman gene expression assays were used for all targets. All targets were standardized to Cyclophilin A (Ppia) signals.

**Histology.** Primary antibodies were purchased from Abcam (K5, K8, GFP), eBioscience (Aire), or Clontech (Living Colors DsRed), and secondary antibodies were purchased from Invitrogen. For Aire-DTR mice, organs were harvested from experimental animals and embedded in Tissue-Tek Optimal Cutting Temperature (OCT) media. 8 um sections were cut and fixed in acetone as described previously.[36] For RFP visualization, whole thymi were fixed 1.5% PFA for 3 hours followed by a 3 hour incubation in 30% sucrose prior to OCT embedding and sectioning. Slides were mounted in Vectashield Hard Mount mounting medium with DAPI (Vector Laboratories) and visualized with either a Zeiss AxioScope2 widefield microscope (2-color imaging) or a Zeiss Apotome widefield microscope (3-color) imaging. Images were analyzed and merged in Photoshop CS3 (Adobe). Linear contrast adjustment was applied equally to all samples and controls. For
mTEC density calculations, widefield images were analyzed with Metamorph. The corticomedullary junction was defined by DAPI and Aire-GFP staining, and cell densities were calculated for the area encompassed by this boundary, as well as for concentric regions moving inward from this boundary by 50 um increments.

Thymic sections for multi-photon imaging were processed by cutting 400 um sections with a vibratome as described previously [59]. Sections were imaged in warm RPMI media bubbled with 95% O₂ and 5% CO₂ using a Mai Tai laser set to 880 nm emission. Images were compiled with ImageJ software and analyzed and rendered with Imaris software.

Organs were examined for lymphocytic infiltrates by formalin fixation and H&E staining as described previously.[40] Lacrimal gland infiltrates were scored for severity of infiltration according to the extent of infiltration and tissue destruction in a blinded manner.[48] Slides were imaged with a Zeiss AxioImager Brightfield microscope.

**Statistical Analysis.** Student’s t-tests (two-tailed with Welch’s correction) were performed using Microsoft Excel and Prism (Graph Pad). Analysis of mTEC densities was performed with a paired t-test.

**Acknowledgements**

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References


Chapter III

eTACs drive CD4+ T cell tolerance through the induction of anergy
Introduction

While *Aire* has a well-characterized role in establishing central tolerance in the thymus, *Aire* expression has also recently been described in a population of extrathymic *Aire*-expressing cells (eTACs) in murine peripheral lymphoid organs. These cells express unique, Aire-regulated TSAs distinct from those driven by thymic Aire, and can cause activation-induced cell death of interacting CD8+ T cells [1]. Other cell populations in the secondary lymphoid organs have also been suggested to promote self-tolerance through intrinsic expression of peripheral TSAs [2-5], and peripheral TSA expression in non-obese diabetic (NOD) mice was recently suggested to correlate chronologically with diabetes progression [6]. Evidence is thus accumulating that expression of both *Aire* and diverse TSAs occurs in the secondary lymphoid organs. However, the ability of any peripheral TSA-expressing population to interact with CD4+ T cells or affect the development of autoimmune disease remains unclear to date.

Here, we find that targeted expression of pancreatic antigens in eTACs robustly prevents CD4+ T-cell mediated autoimmune diabetes. We demonstrate that such tolerance induction is highly resistant to conversion from tolerance to immunogenicity and persists even when cells are transferred serially into susceptible secondary hosts. Finally, we show that the mechanism of eTAC-mediated tolerance depends primarily on the induction of functional inactivation among effectors and not on Treg enrichment, and does so through a mechanism involving deficient costimulation. Together, these results identify eTACs as a unique population of tolerogenic antigen presenting cells.
Results

Transgenic AdBDC mice express a diabetes autoantigen in mTECs and eTACs

Given the high levels of class II MHC expression on mouse and human eTACs, we hypothesized that eTACs might also interact with CD4+ T cells. To investigate this we turned to the BDC2.5 TCR-transgenic mouse model of diabetes [7-9], in which diabetogenic CD4+ T cells react to a pancreatic antigen derived from chromogranin A [10, 11], as well as its mimetope peptide p31 [12]. By inserting the BDC mimetope peptide into the Invariant Chain (Ii or CD74) under direction of the Aire promoter [13], we were able to direct presentation of p31 and expression of GFP to mTECs and eTACs in an Aire-driven BDC antigen (AdBDC) transgenic mouse (Figure 3.1A). Localization of mTECs and eTACs to the thymic medulla and boundary of the T and B cell zones, respectively, was confirmed, as was colabeling of Aire-expressing cells with both Aire protein and GFP (Figure 3.1B). Examination of surface marker expression on GFP+ cells from Adig and AdBDC mice confirmed that Aire-expressing cells identified by the two reporters had identical profiles (Figure 3.1C, 3.1D). Together, these results demonstrate that the AdBDC transgene faithfully recapitulates endogenous Aire expression in mTECs and eTACs.

Targeted expression of BDC antigen in eTACs promotes interaction with cognate CD4+ T cells, and prevents autoimmune diabetes

To test whether eTACs can present antigen to CD4+ T cells, we next employed an adoptive cotransfer system using congenically-marked, CFSE-labeled BDC2.5 CD4+ T
Figure 3.1. The *AdBDC* transgene drives expression of the p31 mimetope peptide in *Aire*-expressing cells. (A) Schematic of *Aire*-driven BDC peptide Invariant Chain-GFP construct targeting the bacterial artificial chromosome (BAC) containing the murine *Aire* locus. The CLIP peptide was replaced with the p31 peptide to facilitate its loading into MHC II molecules of *Aire*-expressing cells. (B) Immunofluorescent staining of *AdBDC* thymus, spleen, and lymph node with anti-GFP (green; all panels), B220 (red; spleen and lymph node left panels), cytokeratin 5 (red; thymus left panel) and anti-Aire (red, right panels) co-staining of thymus, spleen, and lymph node. (C) Representative flow cytometric analysis of GFP and class II expression from wildtype and *AdBDC* NOD thymus, spleen, and lymph node (pre-gated on CD45-low, DAPI-negative). (D) Flow cytometric analysis of DAPI-negative, CD45-low, GFP+ populations from the thymus, spleen and lymph nodes of *Adig* (blue) and *AdBDC* (red) mice for the indicated markers or stained with isotype control (black).
Figure 3.1

A

B

C

D

EpCAM

CD40

PD-L1

PD-L2

CD80

CD86

Thymus

Spleen

Lymph node

AdBDC

Adig

Isotype

0.16

0.24

2.44

0.07

0.72

3.80

0.07

0.72

3.80
cells. Three days post-transfer, BDC2.5 T cells were found to proliferate only in the pancreatic lymph nodes in wildtype recipients. In contrast, robust proliferation of BDC2.5 T cells was observed in all secondary lymphoid organs of AdBDC mice, and by two weeks post-transfer, notable residual populations remained which had completely diluted CFSE (Figure 3.2A). Importantly, proliferation was also observed in AdBDC mice reconstituted with MHCII-/ bone marrow (Figure S3.1A), demonstrating that that direct antigen presentation by radioresistant eTACs is sufficient to drive BDC2.5 T cell proliferation.

We next tested whether cognate antigen expression in eTACs could directly impact BDC2.5-mediated autoimmune diabetes. Purified populations of naïve, CD25-depleted BDC2.5 T cells rapidly cause diabetes in NOD severe combined immunodeficiency (SCID) hosts after adoptive transfer (Figure 3.2B). Strikingly, however, cognate antigen expression on host eTACs in AdBDC SCID recipients led to complete protection from such adoptive transfer of diabetes, and transgenic recipients remained entirely normoglycemic and disease-free (Figure 3.2B). Furthermore, the pancreatic islets of AdBDC SCID mice were devoid of lymphocytic infiltrates or peri-insulitis (Figure 3.2C), suggesting that proliferating BDC2.5 T cells in AdBDC SCID recipients died or remained functionally sequestered prior to reaching the target tissue.

To address whether such tolerogenic function could be achieved by presenting cognate antigen in any non-inflammatory APC population, we also targeted a BDC2.5 mimetope antigen into DEC205+ dendritic cells. To accomplish this we covalently conjugated BDC mimetope antigen to an anti-DEC205 antibody (DEC1040), an approach which has previously used to target antigen to this tolerogenic APC population [14, 15].
Figure 3.2. eTACs in AdBDC mice interact with CD4+ BDC2.5 T cells and prevent induction of autoimmune diabetes in SCID mice. (A) Flow cytometric analysis of CD4+ lymphocyte populations in wildtype (top) and AdBDC (bottom) hosts at 3 (left) and 14 days (right) post adoptive co-transfer of CFSE-labeled Thy1.1+ BDC2.5 T cells and Thy1.2+ polyclonal T cells. (B) Blood sugar values (mg/dl) among NOD SCID mice treated with PBS (green), αDEC1040 (blue) or AdBDC SCID mice (red) following adoptive transfer of BDC2.5 T cells. (C) Hematoxylin and eosin-stained pancreatic islet histology of wildtype and AdBDC SCID adoptive transfer recipients at day 10 post-transfer. Upper panel scale bars = 200 µm, lower panel scale bars = 50 µm.
Figure 3.2

A) Day 3

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Day 14

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<td>AdBDC</td>
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B) Blood glucose (x10² mg/dL)

- WT SCID+PBS (n=5)
- WT SCID+αDEC1040 (n=5)
- AdBDC SCID (n=4)

Days Post-Transfer

0 5 10 15 20

0 1 2 3 4 5 6 7

C) Histological images of WT SCID and AdBDC SCID tissues.
Figure S3.1. Direct antigen presentation by eTACs and anti-DEC1040 treatment both induce robust proliferation of BDC2.5 T cells. (A) Flow cytometric analysis of CD4+ lymphocyte populations in wildtype (top) and AdBDC (bottom) bone-marrow chimeric hosts reconstituted with MHC II-knockout bone marrow at three (left) and fourteen days (right) post-adoptive co-transfer of CFSE-labeled Thy1.1+ BDC2.5 T cells and Thy1.2+ polyclonal T cells. (B) CFSE dilution 40 hours after transfer of labeled CD4+ Thy1.1+ BDC2.5+ T cells into wildtype NOD mice pre-treated 24 hours prior to transfer with PBS (green) or anti-DEC1040 (blue), or into AdBDC mice (red).
Figure S3.1

**A**

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Although this method of delivery differed from the transgenic approach used in AdBDC eTACs, DEC1040-mediated loading of BDC antigen on DCs induced robust proliferation of cognate BDC2.5 T cells (Figure S3.1B). However, such BDC2.5 T cell-DC interactions surprisingly failed to prevent or slow progression to autoimmune diabetes after adoptive transfer into DEC1040-treated NOD SCID recipients (Figure 3.2B). Together, these data indicate that antigen-specific eTAC-CD4+ interactions lead to rapid proliferation and subsequent inactivation of interacting T cells, and result in complete protection from both insulitis and autoimmune diabetes.

eTAC interaction leads to Treg enrichment, but tolerance is Treg-independent

We next attempted to investigate the mechanism of such tolerance by characterizing the phenotype and function of residual eTAC-experienced CD4+ T cells in AdBDC hosts (Figure 3.2A). Despite prolonged interaction with antigen-bearing eTACs, residual BDC2.5 T cells retained significant avidity for I-Ag7-p31 tetramer (Figure 3.3A), suggesting that the surviving T cells in this context had not lost their antigen-specificity or undergone T-cell receptor (TCR) downregulation. These residual BDC2.5 T cells were, however, significantly enriched for Foxp3+ Tregs (Figure 3.3B, 3.3C) despite pre-transfer CD25 depletion. This enrichment was observed most clearly in non-antigen draining secondary lymphoid organs, suggesting that in the absence of interaction with antigen-draining inflammatory APCs in the pancreatic lymph node, eTAC interaction may favor the preferential expansion, retention, or survival of Tregs.
Figure 3.3. Regulatory T cells are dispensable for eTAC-induced tolerance. (A) Flow cytometric analysis of I-Ag7-p31 tetramer avidity among residual Thy1.1+ BDC2.5 T cells at day 14 post-adoptive transfer into wildtype (blue) or AdBDC (red) hosts compared with mock I-Ag7-CLIP tetramer (black). (B) Foxp3 staining of CD4+ Thy1.1+ BDC2.5 T cells taken from spleen, inguinal lymph node (IngLN), or pancreatic lymph node (pLN) 14 days after adoptive transfer into wildtype or AdBDC hosts. (C) Quantitation of (b) showing percentage of Thy1.1+ CD4+ cells that are Foxp3+ (left) and total number of Foxp3+ cells (right) in wildtype (white) or AdBDC (black) recipients. (D) Blood sugar values (mg/dl) among DT-treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of CD4-enriched, DT-treated BDC2.5 Foxp3-DTR T cells, n=3 per condition. (E) Schematic illustration of BDC2.5 serial adoptive transfer strategy. CD25-depleted, CD4-enriched BDC2.5 T cells (naïve) were transferred into primary hosts (WT SCID or AdBDC SCID); at day 10, lymphocytes were re-harvested, purified, and serially transferred into wildtype SCID secondary hosts alone (from primary WT, green, or from primary AdBDC, red) or in a 1:1 mixture with fresh, CD25-depleted, CD4-enriched naïve BDC2.5 T cells (from primary WT hosts with naïve BDC T cells, blue, or from primary AdBDC hosts with naïve BDC T cells, yellow). (F) Diabetes incidence after adoptive transfer among secondary hosts indicated in (E), n=6 per condition.
Figure 3.3

A. Spleen, Ing LN, Panc LN

B. Spleen, Ing LN, Panc LN

C. Graph showing % Foxp3+ T cells and Total # Foxp3+ T cells for WT and AdBDC in Spleen, Ing LN, and Panc LN.

D. Graph showing Blood glucose (x10^2 mg/dL) over Days Post-Transfer for WT SCID + DT and AdBDC SCID + DT.

E. Diagram showing primary and secondary hosts with naive BDC2.5 T cells.

F. Graph showing Percent Non-Diabetic over Days Post-Transfer for different conditions.
To determine whether this peripheral Treg expansion and enrichment explained the mechanism of tolerance induced by eTACs, we next crossed BDC2.5 TCR-transgenic mice with NOD Foxp3-diphtheria toxin receptor (DTR) transgenic mice, which rapidly deplete Foxp3+ Tregs upon exposure to diphtheria toxin [16] (Figure S3.2A). We adoptively transferred BDC2.5+ Foxp3-DTR+ T cells into wildtype or AdBDC SCID mice treated continuously with diphtheria toxin and found that surprisingly, while wildtype SCID hosts progressed rapidly to diabetes, AdBDC SCID recipients remained disease-free (Figure 3.3D). Furthermore, the islets of AdBDC SCID hosts remained devoid of T-cell infiltrates, demonstrating the inability of eTAC-experienced BDC2.5 effector T cells to reach the target organ even in the absence of Tregs (Figure S3.2B). These results suggest that while eTAC-CD4+ T cell interactions support the enrichment of Tregs, such regulatory populations are not required for eTAC-mediated tolerance.

As the activity of Tregs in trans was not critical for eTAC-mediated tolerance of BDC2.5 T cells, we next explored whether effector T cells might be functionally inactivated in cis with a serial co-transfer system. Ten days after adoptive transfer of CD25-depleted BDC2.5 T cells into wildtype and AdBDC SCID hosts, lymphocytes were harvested, purified, and re-transferred into secondary wildtype lymphopenic recipients either alone or mixed 1:1 with freshly isolated naïve, CD25-depleted BDC2.5 T cells (Figure 3.3E). BDC2.5 lymphocytes serially transferred from wildtype SCID mice again induced diabetes in secondary SCID hosts, while serially transferred lymphocytes from AdBDC SCID mice still failed to induce diabetes, despite the absence of continued exposure to cognate antigen-loaded eTACs (Figure 3.3F). Consistent with the results of the Foxp3-DTR experiments above, BDC2.5 T cells harvested from either wildtype or
Figure S3.2. Treg-depleted BDC2.5 T cells fail to infiltrate the pancreas following antigen-specific interactions with eTACs.  (A) Flow cytometric analysis of Foxp3 staining among CD4+ T cells from wildtype and Foxp3-DTR mice treated with a single dose of PBS or DT (50 ug/kg), one day post-treatment. (B) Hematoxylin and eosin-stained pancreatic islet histology of wildtype and AdBDC SCID adoptive transfer recipients at day 10 post-transfer. Treg-depleted naïve Foxp3-DTR BDC2.5 T cells were transferred intravenously and DT was continuously administered.
Figure S3.2

**A**

Flow cytometric analysis of Foxp3 staining among CD4+ T cells from wildtype and Foxp3-DTR mice treated with a single dose of PBS or DT (50 μg/kg), one day post-treatment. The graph shows the percent of maximum Foxp3 expression for different treatment groups:
- WT + PBS
- WT + DT
- Foxp3-DTR + PBS
- Foxp3-DTR + DT

The values are as follows:
- WT + PBS: 8.15
- WT + DT: 9.05
- Foxp3-DTR + PBS: 9.13
- Foxp3-DTR + DT: 0.70

**B**

Hematoxylin and eosin-stained pancreatic islet histology of wildtype and AdBDC SCID adoptive transfer recipients at day 10 post-transfer. Treg-depleted naïve Foxp3-DTR BDC2.5 T cells were transferred intravenously and DT was continuously administered.
AdBDC SCID hosts did not mediate tolerance in trans, as serial co-transfer of purified CD4+ lymphocytes mixed 1:1 with freshly isolated naïve BDC2.5 T cells failed to suppress or delay disease onset (Figure 3.3F). Together, these data suggest that eTAC-mediated tolerance operates primarily in cis—via induction of functional inactivation of effectors—and not in trans—via expansion of Tregs.

eTAC-CD4+ T cell interaction induces functional inactivation via absence of costimulatory signals

To further examine effector T-cell inactivation following eTAC encounter, we adoptively transferred naïve BDC2.5 T cells into AdBDC and wildtype mice, immunized these recipients at day 14 post-transfer with p31 and adjuvant, and measured interferon-γ production three days later (Figure 3.4A). Whereas BDC2.5 T cells isolated from wildtype hosts retained the ability to produce interferon-γ after immunization, BDC2.5 T cells isolated from AdBDC mice remained unresponsive (Figure 3.4B). Likewise, and consistent with the disease transfer results described above, pretreatment of recipient mice with DEC205+ DC-targeted antigen failed to induce anergy upon rechallenge, and indeed led to a more robust secondary recall response (Figure 3.4B), further suggesting that eTACs induce anergy through a cell-type specific mechanism. Consistent with this functional inactivation of effectors, we observed that tolerized T cells expressed high levels of PD-1 and the tolerance-associated markers CD73, FR4, and Lag3 [17, 18], and also had diminished IL-2 production (Figure S3.3A-D).
Figure 3.4. eTACs induce functional inactivation of cognate T cells by presenting antigen in the absence of costimulation. (A) Schematic illustration of p31/CFA immunization protocol to measure recall response of adoptively transferred BDC2.5 T cells. Naïve BDC2.5 T cells were transferred to wild-type, AdBDC, or αDEC1040-treated wild-type mice, and immunized with p31 in complete freund’s adjuvant (CFA) 14 days later. Interferon-γ production to this second stimulation was assessed ex vivo after three days. (B) IFN-γ production among CD4+ Thy1.1+ BDC2.5 T cells isolated from mice indicated in (A); bar graph shows quantitation of pooled data with n=4 for each group. (C) Representative flow cytometric analysis of CD80 staining in DAPI-, CD45+, CD11c+ dendritic cells and DAPI-, CD45-low, MHCII+, GFP+ eTACs in response to anti-CD40/PolyI:C stimulus; quantitation of MFI shifts shown at right. (D) Blood sugar values (mg/dL) among anti-CD40/PolyI:C treated WT SCID and AdBDC SCID hosts after adoptive transfer of 200,000 CD25-depleted, CD4-enriched BDC2.5 T cells, n=4 per condition. (E) Interferon-γ recall responses to a p31 + APC stimulation by CD4-enriched BDC2.5 T cells cultured with AdBDC or p31-pulsed wild-type primary APCs, and with anti-CD28 where indicated. Bar graph shows quantitation of pooled data from three independent experiments. (F) Representative flow cytometric analysis of MHCII expression among by eTACs (green, gated as described previously), cDCs (blue, CD45+ CD11c+), B cells (orange; FSC/SSC, CD19+, CD45+, CD11c-), CD4+ T cells (red; FSC/SSC, CD4+, CD45+, CD11c-), and macrophages (purple; F4/80+ CD45+). (G) Interferon-γ recall responses by CD4+ Thy1.1+ CFSElo BDC2.5 T cells following culture with AdBDC APCs culture in the presence of either anti-I-Ag7 MHC class II blocking antibody or an isotype control. (H) Calcium flux analysis showing kinetic plots of
relative Calcium signaling over time (seconds) of ex vivo BDC2.5 Thy1.1+ T cells adoptively transferred into either wild-type (red) or AdBDC hosts and recovered from non-pancreatic secondary lymphoid organs 14 days later, as detected by Indo-1 dyes, with breaks in the plots occurring during addition of anti-CD3 (30s), cross-linking secondary antibody (60s), and ionomycin (180s) as indicated by arrows. Results are representative of three independent experiments. (I) Flow cytometric of Erk phosphorylation of Thy1.1+ CD4+ T cell populations in (H) after a 30 minute rest period (red) followed by a 2 minute stimulation with anti-CD3 (blue) or PMA (green). Results are representative of three independent experiments.
Figure 3.4

A. naive BDC2.5 T cells

B. WT+ PBS

C. DC

D. Blood Glucose

E. % IFN-γ of CD4+

F. eTACs, cDCs, B cells, M&b T cells

G. % Erk+ of CD4+

H. Relative Calcium Flux

I. WT host AdBDC host

WT host

unstim

CD3

PMA

p-Erk
Figure S3.3. BDC2.5 T cells express tolerance-associated markers following antigen-specific interactions with eTACs. (A) Flow cytometric analysis of PD-1 expression among CD4+ Thy1.1+ BDC2.5 T cells 2 weeks after adoptive transfer into the indicated recipients. Data is representative of results obtained from 5 recipient mice analyzed in two independent experiments. (B) Flow cytometric analysis of CD73 and FR-4 expression among T cells described in (A). (C) Quantitative PCR analyses of the indicated targets for CD4+ Thy1.1+ BDC2.5 T cells 2 weeks after adoptive transfer into the indicated recipients (WT d14 - white, or AdBDC d14 – green), isolated by cell-sorting from non-pancreatic secondary lymphoid organs. Naïve CD4+ BDC2.5 T cells (black) and in vitro activated BDC2.5 T cells (red, bulk splenocytes mixed with 1 ug/mL Ac-p31 in complete DMEM for 72 hr and enriched for CD4+ T cells with a Robosep negative selection kit) were also analyzed for comparison. nd = not detected. (D) Representative flow cytometric analysis of IL-2 and IFN-gamma co-expression by CD4+ Thy1.1+ in draining axial and brachial and non-draining cervical lymph nodes after CFA+p31 immunization 14 days after adoptive transfer, performed in the same manner as Figure 7A-B.
Figure S3.3

(A) Flow cytometric analysis of PD-1 expression among CD4+ Thy1.1+ BDC2.5 T cells 2 weeks after adoptive transfer into the indicated recipients. Data is representative of results obtained from 5 recipient mice analyzed in two independent experiments. (B) Flow cytometric analysis of CD73 and FR-4 expression among T cells described in (A). (C) Quantitative PCR analyses of the indicated targets for CD4+ Thy1.1+ BDC2.5 T cells 2 weeks after adoptive transfer into the indicated recipients (WT d14 - white, or AdBDC d14 – green), isolated by cell-sorting from non-pancreatic secondary lymphoid organs. Naïve CD4+ BDC2.5 T cells (black) and in vitro activated BDC2.5 T cells (red, bulk splenocytes mixed with 1 ug/mL Ac-p31 in complete DMEM for 72 hr and enriched for CD4+ T cells with a Robosep negative selection kit) were also analyzed for comparison. nd = not detected. (D) Representative flow cytometric analysis of IL-2 and IFN-gamma co-expression by CD4+ Thy1.1+ in draining axial and brachial and non-draining cervical lymph nodes after CFA+p31 immunization 14 days after adoptive transfer, performed in the same manner as Figure 7A-B.

IFN-γ/IL-2
To further delineate the mechanisms of eTAC-mediated tolerance, we next interrogated a broad range of established tolerogenic pathways. Notably, blockade of other established inhibitory pathways including PD-L1 and IL-10 failed to break eTAC-mediated tolerance (Figure S3.4A-C). Likewise, we investigated the OX40 alternative costimulatory pathway, which is able to override tolerance in some situations [19]. OX40 is upregulated on BDC2.5 T cells shortly after eTAC encounter (Figure S3.4D) and provision of agonist signal through this receptor can drive an activated phenotype in otherwise tolerized BDC2.5 T cells (Figure S3.4E), although signaling through this pathway was similarly unable to override the diabetes protection observed in AdBDC SCID mice (Figure S3.4F). Because strong costimulation through OX40 engagement could partially override tolerance, we next explored the possibility that the paucity of B7 costimulation on eTACs contributed to their tolerogenic activity [20]. First, we addressed whether the CD80 expression levels on eTACs was sensitive to inflammatory signals, and found that while poly I:C and anti-CD40 caused CD80 upregulation on CD11c+ dendritic cells [14, 15], eTACs appeared unresponsive to these inflammatory signals, as they failed to upregulate CD80 and were still able to induce tolerance in this setting (Figure 3.4C, 3.4D). To determine whether lack of costimulation contributed to eTAC-mediated tolerance induction, we employed an in vitro system of CD4-eTAC interaction in which we used an anti-CD28 agonist. Naïve BDC2.5 T cells were stimulated by either AdBDC APCs, or by p31-pulsed wild-type APCs (Figure S3.5A). Similar to in vivo observations, BDC2.5 T cells became unresponsive to secondary antigen exposure following eTAC interaction, while BDC2.5 T cells stimulated by bulk APCs were able to mount a strong secondary interferon-γ response (Figure 3.4E).
Figure S3.4. eTAC-driven tolerance is resistant to blockade of inhibitory pathways.

(A) Validation of anti-PD-L1 reagent. Blood sugar levels among wildtype NOD mice receiving 200ug/mouse anti-PD-L1 (red) or isotype (blue) beginning at day 0. (B) Blood sugar values (mg/dl) among anti-PD-L1 or isotype treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of 200,000 CD25-depleted, CD4-enriched, BDC2.5 T cells (naïve BDC T cells). (C) Blood sugar values (mg/dL) among anti-IL-10R treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of naïve BDC T cells. (D) Flow cytometric analysis of OX-40 expression on CD4+ Thy1.1+ BDC2.5 T cells 48 hours after adoptive transfer to the indicated recipients. (E) Representative flow cytometric analysis of IL-2 and IFN-γ production by CD4+ Thy1.1+ BDCs 5 days post-transfer to the indicated recipients. OX-40 or isotype was give to recipients on days 0,1,2 and 4 following adoptive transfer. (F) Blood sugar values (mg/dL) among anti-OX40 treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of naïve BDC T cells.
Figure S3.4

(A) Validation of anti-PD-L1 reagent. Blood sugar levels among wildtype NOD mice receiving 200ug/mouse anti-PD-L1 (red) or isotype (blue) beginning at day 0.

(B) Blood sugar values (mg/dl) among anti-PD-L1 or isotype treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of 200,000 CD25-depleted, CD4-enriched, BDC2.5 T cells (naïve BDC T cells).

(C) Blood sugar values (mg/dL) among anti-IL-10R treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of naïve BDC T cells.

(D) Flow cytometric analysis of OX-40 expression on CD4+ Thy1.1+ BDC2.5 T cells 48 hours after adoptive transfer to the indicated recipients.

(E) Representative flow cytometric analysis of IL-2 and IFN-γ-production by CD4+ Thy1.1+ BDCs 5 days post-transfer to the indicated recipients. OX-40 or isotype was given to recipients on days 0, 1, 2 and 4 following adoptive transfer.

(F) Blood sugar values (mg/dL) among anti-OX40 treated wildtype SCID and AdBDC SCID hosts after adoptive transfer of naïve BDC T cells.
Figure S3.5. Anti-I-Ag7 attenuates BDC2.5 T cell interactions with eTACs *in vitro*.

(A) CFSE dilution among naïve Thy1.1+ BDC2.5 T cells co-cultured with wild-type APCs (red), wildtype APCs with non-specific loading of p31 (blue), or AdBDC-derived APCs with p31 expression specifically by eTACs (green). (B) CFSE dilution among naïve Thy1.1+ BDC2.5 T cells co-cultured with AdBDC-derived APCs for 72 hours in the presence of an isotype control (green) or anti-I-Ag7 blocking antibody (purple).
Figure S3.5

(A) CFSE dilution among naïve Thy1.1+ BDC2.5 T cells co-cultured with wild-type APCs (red), wild-type APCs with non-specific loading of p31 (blue), or AdBDC-derived APCs with p31 expression specifically by eTACs (green). 

(B) CFSE dilution among naïve Thy1.1+ BDC2.5 T cells co-cultured with AdBDC-derived APCs for 72 hours in the presence of an isotype control (green) or anti-I-Ag7 blocking antibody (purple).

Figure S8. Anti-I-Ag7 attenuates BDC2.5 T cell interactions with eTACs in vitro. (A) CFSE dilution among naïve Thy1.1+ BDC2.5 T cells co-cultured with wild-type APCs (red), wild-type APCs with non-specific loading of p31 (blue), or AdBDC-derived APCs with p31 expression specifically by eTACs (green). 

(B) CFSE dilution among naïve Thy1.1+ BDC2.5 T cells co-cultured with AdBDC-derived APCs for 72 hours in the presence of an isotype control (green) or anti-I-Ag7 blocking antibody (purple).
Importantly, provision of exogenous costimulation with anti-CD28 caused a partial reversion of the observed anergy in AdBDC cultures, suggesting that lack of CD28 costimulatory signaling during eTAC interaction with antigen-specific T cells contributes to the mechanism of eTAC-mediated antigen unresponsiveness. Given the high levels of MHC class II expression by eTACs relative to other common APC populations (Figure 3.4F), we also investigated the contribution of strong TCR signaling to the induction of tolerance. Interestingly, we found that the addition of MHC class II blocking antibodies to AdBDC cultures could attenuate the peptide:MHC signal delivered by eTACs, as evidenced by reduced cell cycling (Figure S3.5B), and result in increased antigen responsiveness among BDC2.5 T cells that had diluted CFSE (Figure 3.4G). Together, these results suggest that induction of CD4+ T cell tolerance by eTACs involves a combination of factors that include the delivery of a strong TCR stimulus in the absence of effective costimulation.

Finally, to further address the mechanism of BDC2.5 T cell tolerance and unresponsiveness following interactions with eTACs, we examined signaling events downstream of TCR ligation in BDC2.5 T cells following eTAC-driven tolerance induction. BDC2.5 T cells that had encountered eTACs expressing cognate antigen in lymphoreplete AdBDC mice were severely impaired in their ability to increase calcium in response to TCR stimulus (Figure 3.4H), and were also unable to initiate TCR signaling through the parallel Ras/MAPK pathway as evidenced by impaired ERK phosphorylation (Figure 3.4I). These results demonstrate eTAC-tolerized T cells had impaired TCR signaling, at least affecting events downstream of phospholipase C gamma-1 (PLCg1) activation. These results suggest that eTAC interaction induces robust functional
inactivation and antigen unresponsiveness of autoreactive T cells that is highly resistant to conversion from tolerance to immunogenicity.

Discussion

The recent description of *Aire*-expressing cells and ectopic TSA transcription in the secondary lymphoid organs [1-6] has raised the important question of whether intrinsic peripheral self-antigen expression can directly prevent autoimmune disease. Previous descriptions of putatively tolerogenic TSA-expressing populations have focused on interactions with CD8+ T cells [1-5] but given expression of class I MHC on all nucleated cells, it was not clear whether deletion in these systems represented a unique feature of these populations or was merely a byproduct of previously unappreciated antigen expression in non-professional APC populations. Further, the presence, identity, and ontogeny of Aire-expressing populations in the secondary lymphoid organs, and the identity of human eTAC equivalents, have remained unclear. Here we show that eTACs play a unique role among peripheral TSA-expressing populations by interacting with CD4+ T cells. Further, such interactions promote robust, stable T-cell tolerance and prevent progression of autoimmune diabetes.

Consistent with their identity as a distinct professional APC population, we observed that eTACs in transgenic *AdBDC* mice were able to drive extensive proliferation of cognate T cells. Importantly, direct antigen presentation by eTACs, rather than antigen handoff, is sufficient to induce robust CD4+ T cell proliferation.
Remarkably, eTACs induced tolerance among CD4+ BDC2.5 T cells in a SCID setting. While both functional inactivation of effectors and enrichment of Tregs may promote such tolerance, it appears that functional inactivation alone following eTAC:T-cell interactions is sufficient to prevent autoimmunity, as BDC2.5 FoxP3-DTR+ T cells depleted of Tregs remain unable to transfer disease into AdBDC SCID recipients. Also, eTAC-experienced BDC2.5 T cells are unable to transfer disease serially in cis into secondary lymphopenic hosts, and fail to suppress adoptive transfer of disease by other naïve BDC2.5 T cells in trans.

Consistently, tolerized BDC2.5 T cells have impaired signaling TCR signaling leading to both the calcium and Ras/MAPK pathways. The precise site of defective signaling likely involves PLCg1 activation or upstream events. Biochemical characterization of the site of defective signaling is challenging due to the small number of antigen specific unresponsive cells and will require further study. However, the origin of this impaired signaling is not likely simply attributable to high PD-1 expression since co-ligation of PD-1 with the TCR is required for inhibition of TCR signaling [21]. Such antigen unresponsiveness might result from incomplete signaling events through T cells undergoing tolerance induction, such as the delivery of antigen in the absence of costimulatory signals in clonal anergy, but is also observed in conditions of repeated antigen exposure including chronic viral infections, where costimulatory pathways are not specifically impaired [22]. While eTACs are incompetent to deliver a full signal through the CD80/86 costimulatory pathway, persistent self-antigen presentation by eTACs and high MHC class II expression levels may also contribute to their tolerance induction through an exhaustion-like pathway of acquired antigen unresponsiveness in
which costimulatory signaling is not a major factor in tolerance induction [23]. However, the inactivation of Erk signaling we observed in eTAC-tolerized cells was more associated with clonal anergy than exhaustion or adaptive tolerance in a biochemical comparison of models for the two states [24]. Taken together, our results indicate that the primary mechanism of eTAC-mediated disease prevention in this model is recessive—via functional anergy of effectors as opposed to enrichment of Tregs.

Our results also suggest that eTACs may have uniquely pro-tolerogenic capabilities. In support of this, targeting of a BDC mimotope peptide to another putative tolerogenic APC population, DEC205+ DCs, induces proliferation of adoptively transferred BDC2.5 T cells similar to that seen in AdBDC recipients, but fails to protect or delay SCID mice from adoptively transferred diabetes. While the lack of tolerance by DEC1040 was surprising given the established role for immature DEC205+ DCs in tolerance induction [14, 15, 25], dendritic cells from NOD mice may have impaired tolerance [26], and prior studies of DEC205+ DCs have focused on tolerance induction of CD8+ T cells. Also, the lymphopenia in our SCID mice may override tolerogenic DEC205+ DC antigen presentation, as many systems of peripheral antigen display convert from tolerogenic to immunogenic in the context of lymphopenia [27-29]. It should also be mentioned that delivery of antigen via anti-DEC1040 and via the Aire promoter may differ in the dose and length of antigen exposure, which could affect the differential tolerance induction by DEC205+ DCs and eTACs. However, our DEC1040 experiments suggest that eTAC-induced tolerance is not simply a result of the presence of cognate antigen in our disease model, but instead likely depends on additional antigen presentation qualities of eTACs.
Indeed, T-cell tolerance induced upon eTAC encounter appears insensitive to the perturbations that mitigate many other characterized forms of peripheral tolerance. For example, it is striking that self-antigen expression in eTACs prevents autoimmunity even in lymphopenic SCID mice [27-29], and that CD40 and TLR stimuli do not revert eTAC-induced tolerance, in contrast to studies with conventional DC-targeted antigens [14, 15]. Together these findings suggest that self-antigen expression in eTACs induces a remarkably robust T-cell tolerance that is highly resistant to conversion from tolerance to immunogenicity. If indeed eTACs serve as a source of diverse self-antigen expression in the secondary lymphoid tissues of humans, mice, and other mammals, such resistance to immunogenicity may be important in preventing activation of self-reactive T cells. The relative absence of the classical costimulatory molecules CD80 and CD86 on eTACs, the resistance of eTACs to upregulating such molecules in the face of inflammatory stimulus, and the dependence of complete eTAC-mediated tolerance on inadequate costimulation suggest that this mechanism may be central to their role as mediators of peripheral tolerance.

Further, these findings distinguish eTACs among putative TSA-expressing peripheral populations in the secondary lymphoid organs. While expression of individual TSA genes has been reported in fibroblastic reticular cells and lymphatic endothelium, these populations do not express Aire [2-5], and while other transcriptional regulators such as Deaf1 may fulfill this same function [6], the role of any such factor(s) has not yet been linked to TSA expression in a specific population or to the promotion of immunologic tolerance. Further, while the identification of individual candidate TSA genes expressed in such populations may represent genuine antigen production for the
sake of immune education, significant enrichment for a broad range of ectopically expressed TSA genes appears unique to eTACs [1]. This provides an appealing model for complementary tolerance induction to self-reactive T cells that evade adequate negative selection in the thymus, and the fact that such TSA-expressing eTACs can induce tolerance among both CD4+ and CD8+ T cells further supports a broad role for this population in peripheral tolerance.

Together these results demonstrate that targeted antigen expression in eTACs induces robust peripheral self-tolerance through the induction of antigen unresponsiveness. The ability of eTACs to confer such tolerance in secondary hosts represents an attractive therapeutic application for this population in the re-establishment of immune tolerance in both autoimmunity and transplantation.

Materials and Methods

Transgene construction, BAC recombineering, and purification

*Aire*-driven BDC2.5 peptide (*AdBDC*) transgenic mice were generated by standard cloning methods [30] using a bacterial artificial chromosome (BAC) recombineering and transgenesis strategy. The 1040-31 (p31) peptide [YVRPLWVRME; 12] coding sequence was introduced in place of the CLIP sequence of the Invariant Chain using overlapping bi-directional PCR from the pTIM vector (a gift of H. Van Santen) with the forward/reverse primer pairs 5’-AGAATTCTAGAGGCTAGAGCC-3’/ 5’-TCGTACGTACACAGGTTTGGCCAGAT TTC-3’ (product 1) and 5’-
ACGTACGACCACATGGGTACGAATGGAAACGTCAAA TGTCATGGAT-3’/ 5’-
GGCGTGAACTGGAAGATCT-3’ (product 2). These two PCR products were sub-
cloned into pCR-Blunt II TOPO vectors (Invitrogen, Carlsbad, California), sequenced,
and digested with EcoR1 and BsiW1 (product one) or BglIII and BsiW1 (product two),
and ligated sequentially into pLitmus28i (New England Biolabs, Ipswich, Massachusetts)
to generate a complete p31-containing fragment. This EcoR1/BglII fragment was then
inserted into the pTIM vector via EcoR1 partial and BglII complete digest, generating the
pBDCTIM vector. The complete Ii-BDC construct was amplified from pBDCTIM by
PCR using the primers 5’-CGCGGATCCTTAATTAAATGGATGACCAACGCGAC-
3’/5’-CGCGGATCCTCACAGGGTGACTTGACCC-3’, and this product was subcloned
into the pCR-Blunt II TOPO vector for sequencing. This Ii-BDC fragment was inserted
into the BamH1 site of pIRES2-EGFP (Clontech, Mountain View, California), generating
the pli-BDC-IRES2-EGFP vector.

The Ii-BDC-IRES-EGFP fragment was then extracted by PacI/NotI digest and
ligated into pAireFNF, a BAC-targeting plasmid containing 5’ and 3’ homology arms to
the mouse Aire gene. pAireFNF itself was generated from JDFNF, an Aire BAC-targeting
expression construct previously described (Gardner et al., 2008). To generate pAireFNF,
the multiple cloning site from the vector pLitmus 38i (New England Biolabs, Ipswich,
Massachusetts) was amplified with the primers 5’-
GGTTAATTAACTTCTCTGCAGGATATCTG-3’/5’-
GGCCTGCAGGGCCTTGACTAGAGGGTC-3’, subcloned into pCR-BluntII-TOPO,
sequenced, and ligated by NotI partial/HindIII complete digest into JDFNF. Ligation of
the Ii-BDC-IRES-EGFP fragment into pAireFNF generated the construct pAireFNF-Ii-
BDC.

This targeting construct, pAireFNF-Ii-BDC, was then inserted via standard
recombineering [30] into a BAC clone from the Children’s Hospital of Oakland Research
Institute (CHORI) BACPAC Resource Center, clone 461e7 from the RPCI 23 Mouse
BAC Library, which contained the Aire gene and approximately 90Kb of flanking
sequence both upstream and downstream. The BAC identity was previously verified by
PCR and restriction digestion, then purified and transfected via electroporation using
standard techniques into the heat-inducible recombinase-competent and arabinose-inducible Flpe-expressing bacterial strain SW105. The loxP site in the eBAC3.6 vector was first removed by targeted homologous recombination. Heat-induced recombination was achieved by culturing the bacteria at 42°C, rapidly cooling, then electroporating in the presence of linearized pAireFNF-BDC-li plasmid. After selection and PCR-screening of recombinants, Flpe expression was induced in positive clones by culturing in 0.1% arabinose for three hours at 30°C. Flpe-recombinant clones were screened by replicating for acquisition of kanamycin sensitivity. Positive clones were then rescreened by Southern blot using SpeI digest and a 600-bp probe downstream of Aire exon 3 generated by the primers 5’-TCTCGTCCTCAAGAGTGCC-3’ and 5’-GTCATGTTGACGGATCCA-3’.

Highly purified Ii-BDC-Aire BAC DNA was carefully prepared for fertilized oocyte injection by column purification using a modified Midiprep kit protocol (Qiagen, Hilden, Germany). 100ml of clonal liquid culture was pelleted, gently alkaline-lysed, protein-precipitated, and centrifuged. Supernatant was applied to equilibrated Midiprep columns, washed according to manufacturer’s instructions, and eluted with prewarmed 65-degree Buffer QF (Qiagen, Hilden, Germany). BAC DNA was precipitated and pelleted in an ultracentrifuge (Sorvall, Waltham, Massachusetts). Pellets were washed, centrifuged, and allowed to air-dry, then gently resuspended in injection buffer (10mM Tris-HCL, 0.1 mM EDTA, pH 7.5), and evaluated for integrity by KpnI digestion and gel electrophoresis. Resuspended BAC DNA was injected into fertilized NOD oocytes by the UCSF Mouse Transgenic Core with the assistance of H. Lu and N. Killeen.

Mice and genotyping

AdBDC transgenic NOD mice were screened by real-time PCR using the primers 5’-CTGCTGCCCCACAACCA-3’ and 5’-TGTGATCGCGCTTCTCGTT-3’ and the probe Cy5-TACCTGAGCCACCGCTCCCGCT-TMRA along with an endogenous control reaction for CD86 using the primers 5’-CTTGATAGTGTGAATGCCAAGTACCT-3’ and 5’-TGATCTGAACATTGTGAAGTCGTAGA-3’ and the probe FAM-CCGCACGAGCTTTGACAGGAACAACT-TAMRA. AdBDC transgenic mice on the
NOD background were maintained in heterozygosity for all experiments. NOD MHC II-knockout mice were obtained from Jackson Laboratories (stock# 004256). All mice were maintained in microisolator cages and treated in accordance with NIH and American Association of Laboratory Animal Care standards, and consistent with the animal care and use regulations of the University of California, San Francisco.

TCR-transgenic BDC2.5 NOD mice mice were acquired from J. Bluestone and Q. Tang and crossed onto CD90.1 congenic NOD strains. TCR transgenes were maintained in heterozygosity, and were FACS-genotyped by usage of the Vβ4 (BDC2.5) clonotype specific antibody (BD Pharmingen) among peripheral blood CD4s. Foxp3-DTR NOD mice were acquired from D. Mathis and C. Benoist and the Center on Immunological Tolerance in Type-1 Diabetes at Jackson Laboratories.

**Histology and immunofluorescent staining**

Primary and secondary antibodies for mouse histology were purchased from AbCam (Cambridge, United Kingdom), BD Pharmingen (Franklin Lakes, New Jersey), Invitrogen (Carlsbad, California), Jackson ImmunoResearch (West Grove, Pennsylvania), or BioLegend (San Diego, California), with the exception of the anti-Aire antibodies 5C-11 (used for GFP colocalization) and 5H-12 (used for MHC class II colocalization, which were gifts of H. Scott. The 39-10-8 clone which was generated against the I-A<sup>d</sup> allele, but cross-reacts with NOD I-A<sup>G7</sup>, was used for MHC class II detection in mouse lymph node sections. For AdBDC mice, fixation conditions were modified to properly fix cytoplasmic GFP. Organs were directly fixed in a 1.5% paraformaldehyde (Fisher, Waltham, Massachusetts) solution, then transferred to a 30% sucrose solution. GFP was labeled using a Tyramide Signal Amplification kit (Perkin Elmer, Waltham, Massachusetts) according to the manufacturer’s protocol. Slides stained for immunofluorescence were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, California). Anti-B220 (RA3-6B2) and anti-cytokeratin 5 (polyclonal, from AbCam, Cambridge, United Kingdom) were used for GFP co-staining. For hematoxylin and eosin staining, tissues were processed as described previously (Su et
Stained slides were visualized with either an SP2-AOBS confocal microscope (Leica, Wetzlar, Germany) or AxioImager brightfield microscope, and acquired with Leica Confocal Software or AxioVision 4 software (Zeiss, Oberkochen, Germany). Merging, contrast-adjustment, and formatting was applied equivalently to control and experimental images using Photoshop CS3 (Adobe, San Jose, California).

**Flow cytometry and cell sorting**

All FACS antibodies were purchased from BD Pharmingen, eBioscience, Invitrogen, or Southern Biotech with the exception of anti-CD16/32 (clone 2.4G2), which was purified by the UCSF hybridoma core, and anti-EpCAM (clone G8.8), which was purified and biotinylated. eTAC-enriched fractions were prepared for FACS by Percoll gradient centrifugation as described previously [1]. Tetramer, Foxp3, phospho-Erk, and Calcium FACS assays, as well as mRNA transcript analysis by qPCR, was performed as described in Supplemental Experimental Procedures.

Lymphocytes for flow cytometry were prepared by mashing thymi, lymph nodes, or spleens, filtering these cells through a 70µm cell-strainer, lysing red blood cells by incubation in ACK buffer (spleen only; 0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), counting by trypsin blue exclusion, resuspending in anti-Fc receptor blocking antibody clone 2.4G2 in FACS buffer for ten minutes, then incubating on ice with antibody cocktails for twenty minutes. For peptide/class II MHC tetramer staining, cells were incubated on ice with antibody cocktail with either BDC2.5 mimotope/class II MHC tetramer p31-PE (RTRPLWVRME/I-Ag7) or with irrelevant tetramer CLIP-PE (PVSKMRRMATPLLMMQA/I-Ag7, NIAID MHC Tetramer Core Facility) for one hour, then washed and analyzed by flow cytometry. For intracellular Foxp3 staining, lymphocytes were first stained for cell-surface markers, then fixed and permeabilized according to the instructions of the FoxP3 staining buffer set (eBioscience, San Diego, California) and incubated for 30 minutes with anti-Foxp3, washed in permeabilization buffer and then FACS buffer, and analyzed by FACS.
For p-Erk staining, spleen and non-pancreatic lymph nodes were isolated, enriched for CD4+ T cells with a Robosep negative selection kit, and incubated at 37 degrees for 30 minutes to minimize background Erk activation. For CD3 stimulation, anti-CD3 (2C11) was added for 30 seconds, followed by a cross-linking goat anti-hamster antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) for 2 minutes at 37 degrees. PMA stimulated cells were incubated for 2 minutes as a control. Stimulated cells were fixed immediately in BD cytofix (Becton Dickinson, San Jose, California) for 10 minutes at 37 degrees and permeabilized in ice-cold 90% methanol. Fixed cells were stained with an antibody specific to phosphorylated Erk (P-p44/42 MAPK, T202/Y204, Cell Signaling Technologies, Beverly, Massachusetts), followed by staining for surface markers and a secondary APC anti-rabbit antibody (Jackson ImmunoResearch, West Grove, Pennsylvania).

For Ca2+ flux assays, CD4-enriched peripheral lymphoid organ fractions were labeled with Indo-1 (Invitrogen, Carlsbad, California) at 37 degrees for 30 minutes. Labeled cells were then stained for surface markers on ice, and then returned to 37 degrees for 5 minutes prior to analysis. To assess Ca2+ flux, T cell fractions were analyzed on a BD Fortessa (San Jose, California), and kept at 37 degrees for the duration of analysis. Following 30 seconds of data collection, anti-CD3 (2C11) was added and data was collected for another 30 seconds. Next, a cross-linking anti-hamster antibody was added, and events were collected for another 2 minutes. Ionomycin was added at the end of analysis, and events were briefly collected. Calcium flux was analyzed on FlowJo by comparing the kinetics of the Indo-violet/Indo-blue ratio over time.

The following antibody clones were used for FACS experiments: CD4 (RM4-5), CD8(53-6.7), CD45(30-F11), MHC class II (39-10-8), EpCAM (G8.8), CD40 (3/23), PD-L1 (10F.9G2), CD80 (16-10A1), CD86 (GL-1), Thy1.1 (OX-7), Foxp3 (FJK-16s), IFN-gamma (XMG1.2), CD11c (HL3), PD-1 (J43), CD73 (TY/11.8), FR4 (eBio12A5), IL-2 (JES6-1A12), OX-40 (OX-86).
Isolation of RNA and real-time PCR

For real-time PCR, cells from Percoll light fractions were isolated from thymus, spleen, and pooled lymph nodes as described above. eTACs, mTECs, and DCs were sorted from nontransgenic AdBDC mice based on the following surface markers: eTACs and mTECs: PI-, CD45-, CD11c-, MHC II+, GFP+; DCs: PI-, CD45+, CD11c+, MHC II+. RNA was purified using the Absolutely RNA MicroPrep kit (Stratagene, Santa Clara, California) or the Qiagen RNeasy MicroPrep kit (Qiagen, Hilden, Germany) and quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). After quantitation, RNA was reverse-transcribed into cDNA using oligo-dT (Invitrogen, Carlsbad, California) and Superscript III reverse transcriptase (Qiagen, Hilden, Germany). Target expression and cyclophilin A signal (for standardization) were detected using Applied Biosystems gene expression assay primer probe sets. Real-time PCR reactions were run on a 7500 Fast Real-Time PCR System, using ABI Fast Master Mix (Applied Biosystems, Foster City, California).

Purification, CFSE-labeling, and adoptive transfer/serial transfer of T cells

For adoptive transfer of naïve, CD25-depleted, CD4-enriched T cells, spleen and all major peripheral lymph nodes except pancreatic were harvested from nondiabetic donors, pooled by group, ACK-lysed and counted. Cells were CD25-depleted with supernatant from an anti-CD25 hybridoma 7D4 and CD4-enriched using a Mouse CD4+ Negative Selection Kit in a RoboSep (StemCell Technologies, Vancouver, British Columbia, Canada). Aliquots at each step were analyzed to confirm purity. Purified cells were pooled in a ~1:1 ratio of BDC2.5 T cells to polyclonal CD4+ T cells, labeled in 2.5µM CFSE (Invitrogen, Carlsbad, CA) for proliferation experiments, and injected intravenously (IV) at 1-2 x 10^6 cells/mouse in Hank’s Buffered Salt Solution (HBSS) without Phenol Red. 8.3 CD8+ T cells were prepared similarly using a Robosep CD8+ T cell Negative Selection kit. For adoptive transfer of diabetes, 2 x 10^5 unlabeled cells/mouse were injected IV. Serial co-transfer of T cells was performed by harvesting non-pancreatic lymph nodes and spleen from SCID and AdBDC SCID primary recipients.
at day 10. Cells were CD4-purified as described above, and injected to secondary SCID hosts either alone or mixed 1:1 with naïve BDC2.5 T cells at 2 x 10^5 cells/recipient.

**Treatment and analysis of adoptive transfer recipients**

Indicated mice were treated with a 1:1 mixture of anti-CD40 (clone FGK4.5; UCSF Hybridoma Core) and poly I:C (Sigma), or isotype rat IgG (Bio X Cell, West Lebanon, New Hampshire). Mice were injected intraperitoneally one day before and one day after adoptive transfer. Mice receiving *Foxp3-DTR*+ BDC2.5 T cells were treated with 50ug/kg diphtheria toxin (DT; Sigma-Aldrich, St. Louis, Missouri) in PBS injected intraperitoneally the day of adoptive transfer and at day 2 post-transfer, then every three days over the course of observation. Mice were monitored for diabetes using an Ascensia Elite XL blood glucometer (Bayer, Leverkusen, Germany), and considered diabetic after two blood glucose readings >250mg/dl. For αDEC1040 experiments, wildtype NOD mice were injected with 400ng of αDEC1040 or PBS one day prior to transfer of 1 x 10^6 BDC2.5 T cells, and were analyzed for CFSE dilution 40 hours post-transfer. SCID mice were treated with 100ng αDEC1040 or PBS one day prior to adoptive transfer of BDC2.5 T cells, then re-treated with 100ng every three days throughout the course of observation.

Indicated mice were treated with 200ug/mouse anti-PD-L1 (clone 9G2; Bio-Express) or isotype rat IgG (Bio-Express). Mice were injected intraperitoneally one day before and continuously every other day after. For OX-40 blocking experiments, mice were treated with 50 ug of anti-OX-40 (clone OX-86, Bio-X-Cell) at days 0,1,2,4, and 6 post-BDC injection. For IL-10R blocking experiments, 500 ug anti-IL10R (clone 1B1.3A, Bio-X-Cell) was injected on days 1,3,5,7 and 9 following injection of naïve BDCs.

**CFA Immunization Assay**

For peptide/CFA immunization, mice were injected subcutaneously with p31 peptide emulsified in Complete Freund’s Adjuvant (CFA; Sigma-Aldrich, St. Louis,
Missouri) with 4mg/ml H37RA tuberculosis (DIFCO, BD, Franklin Lakes, New Jersey). Acetylated p31 peptide (Genscript, Piscataway, New Jersey) was added to CFA to a final concentration of 500µg/ml. p31/CFA solution was emulsified by sonication, and injected subcutaneously at 100µl/mouse. Three days post-immunization, lymphocytes were harvested, ACK-lysed, and incubated in complete DMEM + 10% FCS plus 0.5 µM p31 peptide. After four hours of incubation, GolgiStop or GolgiPlug (BD Biosciences, Franklin Lakes, New Jersey) was added and cells were incubated an additional six hours before being washed, stained with extracellular marker antibodies, fixed in 4% PFA, incubated in permeabilization buffer (eBioscience, San Diego, California) and stained for intracellular interferon gamma for FACS analysis.

**Generation of αDEC-1040 reagent**

Hybrid antibodies were prepared similarly to previous methods (Hawiger et al., 2001). Plasmid containing the anti-DEC-205 V region heavy-chain linked to the 1040-55 peptide (RVRPLWVRME; Judkowski et al., 2001) were transfected into 293T cells together with a second plasmid containing the kappa chain recognizing DEC-205. 293T cells were maintained in high glucose DMEM containing L-glutamine and 110 mg/L sodium pyruvate, 10% Low IgG FBS, 100U/mL penicillin, 100µg/mL streptomycin, and 50µg/mL gentamicin (all from Gibco, Invitrogen, Carlsbad, California, except gentamicin from Quality Biological, Gaithersburg, MD). Four to six days after transfection supernatants were harvested and antibody was purified over Protein G-sepharose columns (GE Healthcare, Little Chalfont, United Kingdom). Antibodies were dialyzed overnight to PBS and examined for LPS contamination by LAL assay (Lonza, Basel, Switzerland) and for purity by SDS-PAGE and GelCode Blue staining (Pierce, Thermo Scientific, Waltham, Massachusetts). LPS levels below 0.1 EU/mL were considered endotoxin-free. Antibody concentration was determined by spectrophotometric analysis.
**In vitro tolerance assay**

Naïve T cells were prepared according to the adoptive transfer procedure, and mixed with Percoll-enriched APCs isolated from wild-type or *AdBDC* mice by density centrifugation as described previously [1]. 1 x 10^5 naïve BDC2.5 T cells were mixed with 1x10^6 APCs in 24-well plates (Nunc, Roskilde, Denmark), and incubated for 7 days at 37° C in complete DMEM media [32]. Acetylated p31 peptide (YVRPLWVRME, GenScript, Piscataway, New Jersey) ([12] was added control activating conditions at 1 ug/mL. Anti-CD28 (PV-1) was added to indicated cultures at 0.5 ug/mL. Stimulated T cells were isolated by Robosep CD4 enrichment, rested overnight in complete DMEM media, and analyzed for IFN-γ production to a second stimulation with 1 ug/mL p31 and bulk Thy1.2+ splenocytes (described for CFA immunization assay in Supplemental Experimental Procedures). For MHC II blocking experiments, anti-I-A^d^ (clone AG2.42.7, provided by E. Unanue) was used at 10 ug/mL. 2 x 10^4 naïve BDC2.5 T cells were mixed with 2.5 x 10^4 APCs in flat-bottom 96-well plates (Nunc, Roskilde, Denmark), and incubated for 7 days at 37° C in complete DMEM media before being rested overnight and restimulated with PMA/Ionomycin.

**Statistical Analysis**

Statistical analysis of data was performed with Microsoft Excel 2003 and GraphPad Prism 4.0. Statistical comparisons were made using paired t-tests and a two-tailed 95% confidence interval.
References


Chapter IV

eTACs develop from the classical dendritic cell precursor
Introduction

While peripheral Aire expression has been clearly identified by multiple groups [1-3], there have been conflicting reports on the exact identity of the Aire-expressing population in the secondary lymphoid organs. AIRE transcript was found in human lymph nodes during the original identification of AIRE [4], and subsequently detected at the protein level by multiple groups [1, 5, 6]. Investigations into Aire expression in mice also identified Aire transcripts in secondary lymphoid organs [7-9], but came to divergent conclusions regarding the presence of Aire protein in these tissues [7, 8, 10]. Some groups identified peripheral AIRE in cells from the hematopoietic lineage [2, 6], while other investigators have found evidence for Aire expression by CD45− cells in the mouse [3, 11, 12]. Previously, we showed that eTACs express high levels of MHC class II and relevant antigen processing machinery, but lack the high levels of CD80 and CD86 that characterize other antigen presenting populations [3]. While low expression of CD80 and CD86 has also been described for other peripheral TSA-expressing populations [13], expression of MHC class II by these cells appears to be minimal in the absence of inflammatory signals [14].

Here, we identify eTACs as a distinct CD45lo bone marrow-derived APC population, thus reconciling apparently conflicting reports about the identity of eTACs. Furthermore, we find eTACs and conventional dendritic cells express equivalent levels of Zbtb46, a marker of the classical dendritic cell lineage [15, 16]. Finally, we compare surface marker expression by eTACs and conventional DCs, and find that eTACs do not phenotypically resemble any previously described dendritic cell subpopulations, but instead represent a novel lineage of bone marrow-derived antigen presenting cells.
Results

Murine eTACs are a distinct bone marrow-derived APC population

Recent evidence has suggested that peripheral Aire expression maps to a radioresistant cell population [3, 11, 12], but a lack of clarity on distinct markers that are expressed on eTACs has hindered direct analysis of these cells. Utilizing our previously described Aire-reporter mouse (Adig) in which Aire drives expression of GFP and the IGRP antigen [3], we sought to more precisely define this cell population. To map the origin of these cells, we first generated reciprocal bone marrow chimeras and examined the ability of Aire-driven antigen to induce proliferation of adoptively transferred IGRP-specific T cells. Consistent with our previous work we observed that radioresistant cells could drive T-cell proliferation (Figure 4.1A), but also found strong evidence for increased proliferation in wildtype recipients of Adig bone marrow, suggesting that eTACs may be a bone marrow-derived but partially radioresistant population. Importantly, while residual radioresistant eTACs were sufficient to induce 8.3 T-cell deletion as we reported previously [3], eTACs recently generated from the bone marrow were also capable of deleting 8.3 T cells (Figure S4.1A). We next examined peripheral lymphoid organs of these reciprocal chimeric mice by flow cytometry and were surprised to observe that the vast majority of GFP+ CD45lo MHCII+ eTACs were in fact derived from the bone marrow compartment (Figure 4.1B). By immunofluorescence, GFP+ eTACs at the T-B boundary of peripheral lymphoid organs also mapped to the bone marrow compartment (Figure 4.1B), and nuclear Aire staining colocalized with GFP only when the Aire-GFP transgene was expressed by bone marrow-derived cells (Figure 4.1C). However, we did occasionally observe residual Aire-GFP+ eTACs in transgenic

145
Figure 4.1. eTACs are bone marrow-derived. (A) Histograms depicting CFSE dilution among Thy1.1-labeled 8.3 T cells in non-pancreatic lymph nodes three days after adoptive transfer into reciprocal Adig chimeras. (B) Top: representative flow cytometric analysis of peripheral lymphoid organs from reciprocal bone marrow chimeras, made using wildtype and Aire-GFP reporter mice (performed in three independent experiments using either Adig or AdBDC mice, see Fig. 4). Plots are pre-gated on DAPI, CD45lo events. Bottom: Representative immunofluorescent images of lymph node sections from reciprocal chimeras, with Aire-driven GFP (green) and B220 (red) staining. Scale bars = 50 μm. (C) Immunofluorescent detection of Aire protein (red) and Aire-driven GFP (green) in chimeras from (B). Scale bars = 7 μm.
Figure 4.1

A. % of max

B. WT→WT  WT→GFP  GFP→WT  GFP→GFP

C. WT→GFP  GFP→WT

Aire-GFP/B220  Aire-GFP/Aire
**Figure S4.1. Bone marrow-derived eTACs induce 8.3 T-cell deletion.** (A) Flow cytometric analysis of Thy1.1+ 8.3 T cells labeled with CSFE and transferred to the indicated chimeric mice 12 weeks post-reconstitution. CFSE dilution was assessed at 3 and 14 days post-transfer in the indicated secondary lymphoid organs. (B) Aire-GFP (green) and B220 (red) detection by immunofluorescence in lymph nodes taken from a wild-type mouse reconstituted with Aire-GFP transgenic bone marrow. Scale bar = 50 μm.
Figure S1. Bone marrow-derived eTACs induce T-cell deletion. (A) Flow cytometric analysis of Thy1.1+ 8.3 T cells labeled with CFSE and transferred to the indicated chimeric mice 12 weeks post-reconstitution. CFSE dilution was assessed at 3 and 14 days post-transfer in the indicated secondary lymphoid organs. (B) Aire-GFP (green) and B220 (red) detection by immunofluorescence in lymph nodes taken from a wild-type mouse reconstituted with Aire-GFP transgenic bone marrow. Scale bar = 50 µm.
mice receiving wild-type bone marrow (Figure S4.1B), consistent with prior identification of some radioresistance by eTACs [3]. Together, these results demonstrate that eTACs represent a radioresistant bone marrow-derived and not a stromal lineage.

Given these results, we next revisited the expression of the pan-hematopoietic marker CD45 on eTACs. Through the use of additional markers such as EpCAM and CD86, we found that eTACs are not strictly negative for CD45, as reported previously [3, 11, 12], but rather express CD45 at low levels (Figure 4.2A, 4.2B, S4.2A). Interestingly, we analyzed the appearance of eTACs on the stromal cell gating strategy used previously [12] to identify Aire message among gp38− CD31− CD45lo events, and found that eTACs likely fell within this gate due to low but non-negative CD45 expression (Figure 4.2C, 4.2D). We believe this finding explains previous reports of Aire message being detectable in CD45− populations, and establishes eTACs definitively as a bone marrow-derived population.

We next sorted eTACs for comparison to Aire+ mTECs and conventional dendritic cells (cDC), two APC populations with similar transcriptional profiles to eTACs [3], and found that while TSA and Aire expression were highly restricted to eTACs and mTECs, eTACs expressed the characteristic DC markers CD45 and CD11c only at low levels (Figure 4.2B). Importantly, eTACs did express Zbtb46, a recently identified marker of the classical dendritic cell lineage [15, 16], at levels equivalent to splenic cDCs (Figure 4.2B). We also examined eTACs from Zbtb46 reporter mice and found equivalent levels of GFP expression in eTACs and cDCs (Figure 4.2E; [16]. However unlike DCs, eTACs expressed significantly lower levels of CD80 and 86 by qPCR (Figure 4.2F), and did not express detectable levels of most Fc receptors examined.
**Figure 4.2. eTACs are a derived from classical DC precursors.** (A) Gating strategy for identifying splenic eTACs in the absence of a GFP reporter. MHCII⁺ DAPI⁻ events are shown on a CD45 vs. EpCAM plot, and histograms of GFP expression are shown among a distinct EpCAM⁺ CD45lo population. (B) Quantitative PCR results of relative mRNA expression, standardized to cyclophilin, in sorted eTACs, Aire⁺ mTECs (DAPI⁻, CD11c⁻, EpCAM⁺, CD45⁻, MHCII⁺, GFP⁺), and spleen cDCs (DAPI⁻, CD45hi, MHCII⁺, CD11cbi). Left: genes encoding Aire and the tissue-specific antigens Grin2c and Dsg1a. Right: genes encoding Zbtb46, CD45 (Ptprc), and CD11c (Itgax). nd = not detected. Results are representative of at least two independent experiments per target. (C) Flow cytometric analysis of DAPI⁻, FSC/SSC Percoll light fractions from wild-type and Aire-GFP spleen. (D) Back-gating of eTAC events, identified with an equivalent gating strategy to (A), onto the stromal cell identification approach shown in (C). (E) Representative flow cytometric analysis of spleen tissue from Zbtb46-GFP/+ mice showing Zbtb46-GFP expression by an MHC II⁺ pool of CD11c⁻ APCs (red), CD11c⁺ MHC II⁺ cDCs (blue), and eTACs (green) identified as in (A). (F) Additional qPCR analyses of target gene expression by eTACs and cDCs analyzed as in (B).
Figure 4.2
Figure S4.2. Unbiased gating of extrathymic Aire-expressing cells, Fc receptor analysis, and comparison to plasmacytoid dendritic cells. (A) Flow cytometric analysis of DAPI-, FSC/SSC Percoll light fractions from wild-type and Aire-GFP spleens. (B) Quantitative PCR results of relative mRNA expression of the indicated Fc receptor genes, standardized to cyclophilin, in sorted spleen eTACs (DAPI, CD45int, MHCII++, CD86lo, EpCAM+), spleen cDCs (DAPI, CD45hi, MHCII+, CD11chi), CD19+ B cells or CD4+ T cells from the mesenteric LN, spleen macrophages (MO, DAPI-, CD11b+, F4/80+), or mast cells from the peritoneal cavity (DAPI, FceRI+, c-kit+). The absence of bars indicates a lack of detectable transcript for the indicated cell population and Fc target. (C) Plasmacytoid dendritic cell (B220+, PDCA+) analysis from wild-type and Aire-GFP spleens, following Percoll enrichment and DAPI dead cell exclusion.
Figure S4.2

**Figure S2.**

Unbiased gating of extrathymic Aire-expressing cells, Fc receptor expression analysis, and comparison to plasmacytoid dendritic cells.

(A) Flow cytometric analysis of DAPI-, FSC/SSC Percoll light fractions from wild-type and Aire-GFP spleens. (B) Quantitative PCR results of relative mRNA expression of the indicated Fc receptor genes, standardized to cyclophilin, in sorted spleen eTACs (DAPI-, CD45int, MHCII++, CD86lo, EpCAM+), spleen cDCs (DAPI-, CD45hi, MHCII+, CD11chi), CD19+ B cells or CD4+ T cells from the mesenteric LN, spleen macrophages (MO, DAPI-, CD11b +, F4/80 +), or mast cells from the peritoneal cavity (DAPI-, FceRI +, c-kit +). The absence of bars indicates a lack of detectable transcript for the indicated cell population and Fc target. (C) Plasmacytoid dendritic cell (B220+, PDCA+) analysis from wild-type and Aire-GFP spleens, following Percoll enrichment and DAPI dead cell exclusion.
(Figure S4.2B), suggesting a limited potential internalize and present opsonized foreign antigen. Of note, eTACs and DCs both expressed detectable levels of the transcriptional regulator Deaf-1, which had recently been associated with TSA expression in bulk secondary lymphoid organs (Figure 4.2F), although the transcriptional variant of Deaf-1 identified by Yip et al. was undetectable in sorted cell populations (2009). Finally, sorted eTACs also displayed morphological features similar to dendritic cells, namely large, highly vacuolated cytoplasm (Figure 4.3A). To further characterize the relationship of cDCs and eTACs, we then examined a panel of surface markers characteristic of cDCs (Figure 4.3B) and plasmacytoid DCs (Figure S4.2C), and confirmed that eTACs fall into neither group, and appear to represent a distinct, non-conventional APC population.

**eTACs in human secondary lymphoid organs**

To identify and characterize peripheral Aire-expressing populations in humans, we examined lymph node sections by immunofluorescence and were able to identify discrete cells expressing intranuclear Aire protein (Figure 4.4A) in all patient samples examined (6/6). Further, as in mouse, human eTACs were uniformly MHC class II+, and localized to the subcapsular zone at the boundary between the T cell paracortex and B cell follicles within the lymph node (Figure 4.4A). Importantly, we found that Aire protein in human eTACs and mTECs was exclusively intranuclear and concentrated in nuclear speckles (Figure 4.4B, S4.3A, S4.3B), similar to the localization of transcriptionally active Aire protein in the thymus [17]. As in mice [3], Figure 4.2), human eTACs did not stain
Figure 4.3. eTACs are a distinct type of antigen presenting cell. (A) Giemsa staining of sorted eTACs (top) and conventional DCs (bottom) from the spleens of Adig donors, cytospun onto slides, and visualized by light microscopy. eTACs: FSC/SSC, DAPI−, CD45lo, MHCIIhi, CD86−, Aire-GFP+. cDCs: FSC/SSC, DAPI+, CD45hi, MHCII+, CD11chi. (B) Representative histograms from flow cytometric analysis of the indicated markers on eTACs (green), CD45- stromal cells (red), and cDCs (blue).
Figure 4.3

A

eTACs

cDCs

B

CD45-stroma
cDC
eTAC

CD4
CD8a
CD11b
CD11c
CD40

CD45
CD80
CD103
CCR7
F4/80
Figure 4.4. eTACs are present in human lymph nodes. (A) Immunofluorescent staining of eTACs from mouse and human lymph nodes for MHC class II (green) and Aire (red) and counterstained with DAPI (blue), showing localization of eTACs to the T cell zone near B cell follicles. Note the rare Aire-positive/Class II positive cells in the T cell zone of both human and mouse lymph node sections (arrows). The outline of the follicle (Fo) is highlighted by a line for orientation. Scale bar = 50 µm (B) Representative high-power confocal images of mouse and human eTACs showing nuclear Aire speckling. Scale bar = 10 µm. (C) Representative confocal images of eTACs with Aire (red) and co-staining of CD45 (left, scale bar = 10 µm), CD11c (center, scale bar = 25 µm), and CD11b (right, scale bar = 25 µm).
Figure S4.3. Aire localizes to nuclear speckles in mouse and human medullary thymic epithelial cells. (A) Aire protein (red) is visualized by immunofluorescence, and is seen in a subset of MHC class II+ cells (green) in the thymic medulla. Scale bars = 25 μm. (B) A zoomed in view of the area indicated in (A) shows Aire is present within DAPI+ (blue) nuclei. Scale bars = 10 μm.
Figure S4.3

Aire localizes to nuclear speckles in mouse and human medullary thymic epithelial cells. 

(A) Aire protein (red) is visualized by immunofluorescence, and is seen in a subset of MHC class II+ cells (green) in the thymic medulla. Scale bars = 25 µm.

(B) A zoomed in view of the area indicated in (A) shows Aire is present within DAPI+ (blue) nuclei. Scale bars = 10 µm.
strongly for the conventional dendritic cell markers CD11c and CD45, and also lacked CD11b (Figure 4.4C). Together, these results identify a discrete population of eTACs in human secondary lymphoid organs closely resembling their murine counterparts.

Discussion

Here we show that mouse and human secondary lymphoid organs contain similar populations of discrete extrathymic Aire-expressing cells and that such cells represent a novel bone marrow-derived population of APCs that derive from the classical dendritic cell precursor. Our investigation into the origin of eTACs led us to the unexpected conclusion that eTACs are in fact not a stromal population as suggested previously, but are rather bone marrow-derived. The lack of high levels of CD45 and APC markers such as CD11c among cells labeled with the Adig reporter, combined with evidence of radioresistance in adoptive transfer experiments with cognate T cells, had originally indicated that at least some Aire-expressing cells in the peripheral lymphoid tissues were radioresistant stromal cells, although initial experiments had also shown that peripheral Aire-expressing cells could develop from the bone marrow [3]. A comparison of the relative induction of cognate T cell proliferation in reciprocal Adig chimeras in which Aire-driven antigen was restricted to either the radioresistant or bone-marrow derived compartment showed that the bone-marrow restricted condition most closely replicated initial observations in unmanipulated Adig mice [3]. Further analysis of bone marrow chimeras made with both the Adig and AdBDC reporters of Aire expression revealed that the bone marrow compartment was the primary source of Aire-driven GFP in these mice, demonstrating that eTACs are bone-marrow derived, and that a degree of radioresistance
by eTACs explains their detection in the radioresistant compartment. The possibility also remains that authentic radioresistant stromal cell types express Aire at low levels and explain our detection of Aire-driven antigen in this compartment, but CD45$^{hi}$ MHCII$^{hi}$ CD80/86$^{lo}$ EpCAM$^{hi}$ eTACs appear to be the primary source of Aire in the periphery, and the use of an improved eTAC gating strategy allowed us to identify equivalent levels of Aire expression among sorted peripheral eTACs and Aire+ mTECs from the thymus.

The identification of eTACs as a bone-marrow derived population led to the question of their relationship to other APC populations. The original identification of eTACs noted their apparent lack of the cDC marker CD11c, as well as markers of other APC lineages. Here we show that eTACs express CD45 at low levels, consistent with their development from the bone marrow. We also identified CD11c expression at low levels in eTACs, as reported by Poliani et al. in peripheral human lymph nodes (2010). Furthermore, we found that eTACs and conventional DCs expressed equivalent levels of the recently identified transcription factor Zbtb46 [15, 16], which suggests that eTACs develop through the committed classical DC precursor. Nevertheless, a comparison of surface marker expression between eTACs and conventional DCs shows that while eTACs have a homogenous FACS profile with respect to the markers investigated, they do not clearly fall into any previously identified subset of dendritic cells, supporting the conclusion that eTACs are a distinct and previously undescribed population of APCs.

Together these results demonstrate that eTACs are a distinct type of bone marrow-derived APC. Our findings that eTACs are a novel bone-marrow derived population from the classical DC lineage should facilitate the identification and development of in vitro differentiation and expansion conditions that will make
therapeutic eTAC transfers feasible for the induction of antigen-specific immune tolerance.

Materials and Methods

Mice and genotyping

Adig transgenic NOD mice[3] were screened by real-time PCR with the same reaction as AdBDC mice (Chapter 3 methods) as it detects genomic GFP present in both transgenes. Both Adig and AdBDC transgenes were maintained in heterozygosity for all experiments. TCR-transgenic 8.3 NOD mice were acquired from J. Bluestone and Q. Tang and crossed onto CD90.1 congenic NOD strains. 8.3 mice were maintained in heterozygosity, and were FACS-genotyped by usage of Vβ8.1 clonotype specific antibodies (BD Pharmingen) among peripheral blood CD8s. 129S6/SvEv.Zbtb46-GFP mice have been previously described [16]. All mice were maintained in microisolator cages and treated in accordance with NIH and American Association of Laboratory Animal Care standards, and consistent with the animal care and use regulations of the University of California, San Francisco.

Flow cytometry and cell sorting

All FACS antibodies were purchased from BD Pharmingen, eBioscience, Invitrogen, or Southern Biotech with the exception of anti-CD16/32 (clone 2.4G2), which was purified by the UCSF hybridoma core, and anti-EpCAM (clone G8.8), which was purified and biotinylated. eTAC-enriched fractions were prepared for FACS by Percoll gradient centrifugation as described previously (Gardner et al., 2008).
Lymphocytes for flow cytometry were prepared by mashing thymi, lymph nodes, or spleens, filtering these cells through a 70μm cell-strainer, lysing red blood cells by incubation in ACK buffer (spleen only; 0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), counting by trypan blue exclusion, resuspending in anti-Fc receptor blocking antibody clone 2.4G2 in FACS buffer for ten minutes, then incubating on ice with antibody cocktails for twenty minutes.

The following antibody clones were used for FACS experiments: CD4 (RM4-5), CD8(53-6.7), CD11b (M1/70), CD45(30-F11), MHC class II (39-10-8), EpCAM (G8.8), CD40 (3/23), PD-L1 (10F.9G2), CD80 (16-10A1), CD86 (GL-1), Thy1.1 (OX-7), CD103 (2E7), CCR7 (4B12), F4/80 (BM8), B220 (RA3-6B2), CD11c (HL3).

**Reciprocal bone marrow chimeras**

Wild-type and Aire-GFP reporter mice (*Adig* for 8.3 adoptive transfers, *AdBDC* mice for the displayed Aire-GFP plots) were used as donors and/or recipients to generate chimeric mice in order to restrict transgene expression to the bone marrow, radioresistant host, neither, or both compartments. Recipient mice received two irradiation cycles totaling 1300 rads (900 in first dose, 400 in second), with at least 3 hours between the two doses. After the first dose, donor bone marrow was harvested and depleted of T cells as described previously (Gardner et al., 2008). Bone marrow was injected i.v. at 1 x 10⁷ cells per host, and mice were harvested after at least 8 weeks of reconstitution.

**Isolation of RNA and real-time PCR**

For real-time PCR, cells from Percoll light fractions were isolated from thymus, spleen, and pooled lymph nodes as described above. eTACs, mTECs, and DCs were sorted from nontransgenic *AdBDC* mice based on the following surface markers: eTACs and mTECs: PI-, CD45-, CD11c-, MHC II+, GFP+; DCs: PI-, CD45+, CD11c+, MHC II+. RNA was purified using the Absolutely RNA Microprep kit (Stratagene, Santa Clara, California) or the Qiagen RNeasy Microprep kit (Qiagen, Hilden, Germany) and quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham,
Massachusetts). After quantitation, RNA was reverse-transcribed into cDNA using oligo-
dT (Invitrogen, Carlsbad, California) and Superscript III reverse transcriptase (Qiagen,
Hilden, Germany). Target expression and cyclophilin A signal (for standardization) were
detected using Applied Biosystems gene expression assay primer probe sets. Real-time
PCR reactions were run on a 7500 Fast Real-Time PCR System, using ABI Fast Master
Mix (Applied Biosystems, Foster City, California).

Histology and immunofluorescent staining

Primary and secondary antibodies for mouse histology were purchased from
AbCam (Cambridge, United Kingdom), BD Pharmingen (Franklin Lakes, New Jersey),
Invitrogen (Carlsbad, California), Jackson ImmunoResearch (West Grove, Pennsylvania),
or BioLegend (San Diego, California), with the exception of the anti-Aire antibodies 5C-11 (used for GFP colocalization) and 5H-12 (used for MHC class II colocalization, which
were gifts of H. Scott. For Adig mice, staining was performed as described previously
(Gardner et al., 2008). The 39-10-8 clone which was generated against the I-A\(^d\) allele, but
cross-reacts with NOD I-A\(^b\), was used for MHC class II detection in mouse lymph node
sections. For AdBDC mice, fixation conditions were modified to properly fix
cytoplasmic GFP. Organs were directly fixed in a 1.5% paraformaldehyde (Fisher,
Waltham, Massachusetts) solution, then transferred to a 30% sucrose solution. GFP was
labeled using a Tyramide Signal Amplification kit (Perkin Elmer, Waltham,
Massachusetts) according to the manufacturer’s protocol. Slides stained for
immunofluorescence were mounted in Vectashield mounting medium with DAPI (Vector
Laboratories, Burlingame, California). Anti-B220 (RA3-6B2) and anti-cytokeratin 5
(polyclonal, from AbCam, Cambridge, United Kingdom) were used for GFP co-staining.
For hematoxylin and eosin staining, tissues were processed as described previously (Su et
al., 2008). Stained slides were visualized with either an SP2-AOBS confocal microscope
(Leica, Wetzlar, Germany) or AxioImager brightfield microscope, and acquired with
Leica Confocal Software or AxioVision 4 software (Zeiss, Oberkochen, Germany).
Merging, contrast-adjustment, and formatting was applied equivalently to control and
experimental images using Photoshop CS3 (Adobe, San Jose, California).
All of the human tissue that was obtained for evaluation in this study was obtained with approval from and under the guidelines of the UCSF Committee on Human Research. Human thymic tissue was obtained from the San Francisco General Hospital Pathology Core. Formalin-fixed, paraffin-embedded sections of human pancreatic and non-pancreatic lymph nodes from non-diabetic control patients were obtained from the Network for Pancreatic Organ Donors with diabetes (nPOD). After deparaffinizing and rehydrating, heat-induced antigen unmasking was performed using 1mM EDTA (Poliani et al., 2010). Endogenous peroxidase activity was quenched with 1% H₂O₂ in PBS. Staining for AIRE was done using the anti-human AIRE antibody clone 6.1 (a generous gift from P. Peterson) and the TSA plus TMR kit (Perkin Elmer, Waltham, Massachusetts). The secondary was either a goat anti-mouse IgG-biotinylated antibody (Vector, Burlingame, California) followed by streptavidin-HRP or the Vector anti-mouse Ig ImmPress polymer reagent. Co-stains were done after AIRE-staining was complete. For MHC class II, sections were first blocked with the Mouse-on-Mouse (M.O.M.) reagent (Vector, Burlingame, California), stained with anti-HLA-DP, DQ, DR (Dako, Glostrup, Denmark), and detected using a FITC-conjugated goat-anti-mouse IgG Fab fragment (Jackson ImmunoResearch, West Grove, Pennsylvania). We also used the following antibodies for co-staining: CD11c (EP1347Y), CD11b (EP1345Y), CD45 (HI30).

Purification, CFSE-labeling, and adoptive transfer/serial transfer of T cells

For adoptive transfer of CD8-enriched 8.3 T cells, spleen and all major peripheral lymph nodes except pancreatic were harvested from nondiabetic donors, pooled by group, ACK-lysed and counted. Cells were CD8-enriched using a Mouse CD8+ Negative Selection Kit in a RoboSep (StemCell Technologies, Vancouver, British Columbia, Canada). Aliquots at each step were analyzed to confirm purity. Purified cells were pooled in a ~1:1 ratio of 8.3 T cells to polyclonal CD4+ T cells, labeled in 2.5μM CFSE (Invitrogen, Carlsbad, CA) for proliferation experiments, and injected intravenously (IV) at 1-2 x 10⁶ cells/mouse in Hank’s Buffered Salt Solution (HBSS) without Phenol Red.
References


Chapter V

Conclusion
T cell receptor diversity is generated through random recombination processes that lead to creation of T cell receptors capable of recognizing a broad spectrum of antigens in the form of processed peptides. Selection processes operate in the thymus to remove or skew the activity of developing T cells, and similar processes also act upon mature T cells in the periphery. While many proteins are expressed ubiquitously and can serve as a source of antigen to drive removal of autoreactive clones in the thymus and periphery, other antigens such interphotoreceptor retinoid-binding protein (IRBP) are expressed only in the retina [1]. The restricted expression of this and other antigens, combined with the immunological privilege or sequestration of antigens in sites such as the eye, presents a need to expose developing T cells to these tissue-restricted or tissue-specific antigens (TSAs) in a tolerogenic manner prior to their release to peripheral tissues. While Aire-independent TSAs have been documented [2], Aire is responsible for driving expression of a large number of tissue-specific antigens in the thymus [3], and the need for such processes is demonstrated by the multi-organ autoimmunity that occurs when the normal function of Aire is disturbed through mutations [4, 5]. Aire expression has been mapped to a subset of medullary thymic epithelial cells (mTECs) characterized by high CD80 and MHC II expression [6, 7]. However, the developmental potential of Aire+ mTECs has been unclear [7, 8], and there has been debate about whether Aire directly or indirectly affects the expression of TSAs [9, 10]. Aire is also expressed in the periphery [3], although the source of peripheral Aire has been ascribed to both stromal and hematopoietic populations [11-13]. Furthermore, while Aire also drives the expression of TSAs in the periphery [11], the contribution of peripheral Aire to tolerance induction has been unclear.
**Aire+ mTECs undergo rapid turnover and progress to a post-Aire stage**

In Chapter II, we performed a series of experiments with two novel transgenic mouse lines to address the recovery and developmental potential of Aire+ mTECs. We generated an Aire-DTR mouse to allow temporal Aire+ mTEC ablation, and found that while a single injection of diphtheria toxin (DT) led to specific ablation of Aire+ mTECs, repeated DT injections also led to loss of Aire- mTEC subsets. Broad ablation of mTECs through repeated DT ablation quickly led to defective central tolerance, which we observed most dramatically in the RIP-mOVA x OT-II system of TEC-dependent selection [14]. Loss of Aire- mTECs contrasted with a model of Aire+ mTEC development put forth by Gray et al., where, based on the observation that Aire+ mTECs were not actively dividing and that Aire itself could drive apoptosis in vitro, they suggested that Aire expression was the last step in a linear mTEC differentiation pathway [7]. However, the apparent off target ablation of Aire- mTECs in repeatedly treated Aire-DTR mice could be a result of several of mechanisms. Thus we chose to directly test the hypothesis that Aire+ mTECs could differentiate into other mTECs, and to do so, we generated an inducible Aire-Cre mouse, which, when cross with a Rosa-RFP allele, permitted irreversible labeling of Aire+ mTECs for use in fate mapping experiments, an approach which has been used previously to perform fate mapping experiments with Foxp3+ regulatory T cells [15]. These lineage-tracing experiments provided evidence that Aire+ mTECs could indeed progress to a post-Aire stage of development, although we found that such cells did not contain stem cell potential, and were ultimately replaced from a non-Aire-expressing TEC population (Figure 5.1).
Figure 5.1. mTECs dynamically progress through stages of induction and repression of Aire. Aire$^+$ mTECs develop from an Aire- progenitor population, which expresses low amounts of MHC class II and lacks TSA expression. Aire$^+$ mTECs can also progress to a post-Aire state that is similarly low for MHC class II retains intermediate levels of TSA expression.
Figure 5.1

- Aire
- Tissue-specific
- self antigens
- MHC
- class II

Aire\(^{+}\) mTEC

Thymus

Pre-Aire mTEC

Post-Aire mTEC
Suggestions of a post-Aire stage of mTEC development have existed in the literature for several years, but prior experimental systems did not conclusively document the existence of this cell population. Nishikawa et al. used an Aire-driven Cre-inducible reporter system to track the fate of Aire+ cells, but their approach permitted constitutive Cre activity, and in many of their founder mice, they observed ubiquitous labeling resulting from a brief wave of Aire expression during embryogenesis [8]. They were able to obtain founder mice with reporter expression specifically in mTECs, although these mice seemed to have a low level of reporter activity. Nevertheless, they came to similar conclusions as our findings, namely, that Aire+ mTECs can lose expression of both Aire and maturation markers. Other papers have addressed post-Aire development by staining for involucrin [16, 17], a marker associated with late stages of skin differentiation and suggested to mark terminally-differentiated, post-mitotic mTECs. However, with our inducible Aire-Cre mice, we were able to directly identify post-Aire mTECs and find that they were substantially enriched for involucrin expression. Similarly, we were able to assess TSA expression in Aire+ and post-Aire mTECs, and through our observation that TSAs were expressed highly in Aire+ mTEC and moderately in post-Aire mTECs, we concluded that Aire likely has a direct role in initiating the transcription of TSAs. The persistence of TSA expression beyond Aire may simply reflect a temporal lag between the termination of Aire expression and its TSA-inducing function, or it may result from the persistence of non-Aire TSA-inducing co-factors that Aire recruits to the sites of transcription [9]. Regardless of the mechanism of TSA persistence, the existence of TSAs in MHCIIlo CD80lo post-Aire mTECs suggests that antigen presentation by these mTECs have a non-redundant role with that carried out by the MHCIIhi CD80hi Aire+
subset, and future experiments should seek to identify this role. Additional work should also focus on identifying the stem cell population which gives rise to Aire$^+$ mTECs, and our lineage tracing system may be useful in separating pre- and post-Aire mTECs with immature surface marker phenotypes.

**Extrathymic Aire-expressing cells drive CD4$^+$ T cell tolerance**

Previous work from our lab had used an Aire-driven IGRP-GFP (Adig) mouse to force expression of the diabetes autoantigen Islet-specific glucose-6-phosphatase related protein (IGRP) in extrathymic-Aire expressing cells (eTACs), and found that IGRP-specific 8.3 T cells underwent dramatic expansion and deletion following encounters with eTACs in these mice [11]. Through phenotyping of this novel peripheral APC population, it was found that eTACs expressed high levels of MHC class II, in contrast to other peripheral stromal populations which have been suggested to contribute to tolerance, and thus we sought to determine whether or not eTACs might also be capable of driving tolerance to CD4$^+$ T cells. Towards this end, we generated an Aire-driven BDC antigen mouse, which expressed an MHC II-restricted diabetes autoantigen recognized by BDC2.5 CD4$^+$ TCR transgenic T cells specifically in Aire$^+$ cells. We first validated the ability of eTACs in AdBDC mice to interact with BDC2.5 T cells, and found that while BDC2.5 T cells proliferated extensively following AdBDC eTAC encounters, the CD4$^+$ T cells did not undergo deletion to the same extent as the CD8$^+$ T cells in the Adig:8.3 system, and a substantial proportion of expanded CD4$^+$ T cells remained two weeks after transfer (Figure 5.2). We assessed the function of these remaining BDC2.5 T cells and found that they had become anergic and failed to secrete
Figure 5.2. eTACs drive anergy of CD4$^+$ T cells and deletion of CD8$^+$ T cells.

Illustration of the alternative fates of CD4$^+$ and CD8$^+$ T cells following eTAC encounter. While antigen-specific encounters with eTACs results in extensive proliferation of both CD4$^+$ and CD8$^+$ T cells, only CD8$^+$ T cells are completely deleted; CD4$^+$ populations undergo some contraction after their initial burst of proliferation, but a significant pool of anergic, antigen-experienced CD4$^+$ T cells persists for at least two weeks following initial interactions with eTACs.
Figure 5.2

![Diagram showing antigen encounter drives proliferation and leads to anergy or deletion.](image)

- eTAC
- CD4+ T cell
- CD8+ T cell
- Aire
- Tissue-specific self antigens
- MHC class II
- MHC class I

antigen encounter drives proliferation

anergy

deletion
IFN-γ in response to immunization, exhibited impaired calcium signaling, and failed to phosphorylate Erk in response to TCR stimulation. We repeated our adoptive transfer experiments with mice on the SCID background, and found that while wild-type SCID mice developed diabetes following BDC2.5 transfer, AdBDC mice were completely protected from disease induction, and such protection depended on induction of anergy among effectors rather than active Treg suppression.

While our experiments comparing AdBDC and wild-type mice demonstrated that antigen expression by eTACs was sufficient to drive tolerance, it was unclear whether such tolerance induction was unique to eTACs or might also be induced by other APCs. Thus, we performed BDC2.5 adoptive transfer experiments in wild-type mice treated with anti-DEC205-conjugated BDC2.5 antigen, an approach that delivers antigen specifically to the DEC205⁺ subset of dendritic cells [18, 19]. Through these experiments, we found that DEC205-delivered antigen was insufficient to recapitulate the tolerance observed with eTAC-presented antigens, which suggested that eTAC-driven tolerance might depend on unique, cell-intrinsic factors expressed by eTACs. Interestingly, eTACs are distinct from many other professional APCs in their lack of the B7 costimulatory molecules CD80 and CD86, and through a series of in vitro experiments in which missing B7 costimulation was replaced with an agonistic antibody directed towards the B7 receptor CD28, we found that tolerogenic antigen presentation by eTACs depended at least in part on the lack of CD80 and CD86 on eTACs. Taken together, these results support the hypothesis that tolerance induction by eTACs depends on eTAC-specific antigen presentation capabilities. Future work should seek to identify additional costimulatory pathways utilized by eTACs to present antigen in a tolerogenic
manner. Also, while these proof-of-principle experiments demonstrate that eTACs are able to drive CD4<sup>+</sup> T cell tolerance and provide some mechanistic insight into how such tolerance is established, future work should also seek to identify the contribution of eTACs to peripheral tolerance induction in a more physiological setting of autoimmune disease progression.

**Extrathymic Aire-expressing cells are a distinct bone marrow-derived population**

Aire expression in the periphery was initially reported in CD45<sup>−</sup> stromal cells [20], and experiments in our lab with an Aire-GFP reporter also identified Aire expression among a CD45<sup>−</sup> stromal population, although bone marrow chimera experiments with Aire-GFP reporter mice provided evidence for Aire expression in both the hematopoietic and radioresistant compartments [11]. To determine the origin of eTACs, we performed a series in reciprocal bone marrow chimeras and found that peripheral Aire expression reliably mapped the bone marrow compartment, although there was some level radioresistant signal as well. We revisited expression of the canonical hematopoietic marker CD45 by eTACs, and found that eTACs expressed detectable amounts of CD45, and have since characterized CD45 expression by eTACs as low or intermediate. We also examined an extensive panel of DC-associated surface markers such as CD11c, and found that, to our knowledge, eTACs did not fit any previously defined subset of dendritic cells, but appeared to represent a novel population of peripheral APCs. Interestingly, we found that eTACs expressed high amounts of Zbtb46, a recently described marker of dendritic cells which develop from the classical
dendritic cell precursor [21, 22], which further suggests that eTACs are an atypical subpopulation of dendritic cells. This developmental pathway should provide an avenue for the *in vitro* culture and expansion of eTACs, since several subsets of dendritic cells can be effectively cultured and expanded with pathways such as Flt3 ligand [23]. Future work should seek to establish *in vitro* developmental conditions for eTAC differentiation and expansion, which will aid in experiments assessing eTAC function *in vivo*, and may provide a means for eTAC generation and adoptive transfer in therapeutic applications.

**References**


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