

# The Cellular Mechanism of Aire Control of T Cell Tolerance

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## Summary

Aire promotes the tolerization of thymocytes by inducing the expression of a battery of peripheral-tissue antigens in thymic medullary epithelial cells. We demonstrate that the cellular mechanism by which Aire exerts its tolerance-promoting function is not primarily positive selection of regulatory T cells, but rather negative selection of T effector cells. Surprisingly, supplementing its influence on the transcription of genes encoding peripheral-tissue antigens, Aire somehow enhances the antigen-presentation capability of medullary epithelial cells. Thus, this transcriptional control element promotes central tolerance both by furnishing a specific thymic stromal cell type with a repertoire of self antigens and by better arming such cells to present these antigens to differentiating thymocytes. In Aire's absence, autoimmunity and ultimately overt autoimmune disease develops.

## Introduction

A number of central and peripheral mechanisms of T cell tolerance have been identified based primarily on results on genetically engineered mouse models (reviewed in [Ohashi, 2003](#); [Walker and Abbas, 2002](#)). An outstanding issue is how these different mechanisms integrate to enforce tolerance in unmanipulated mice and humans: which of the diverse tolerization modes dominate; which are experimental anomalies; to what extent are they redundant versus interdependent; and how do their roles vary with genetics, environment, or age? One approach to addressing such questions about how a tolerant state is achieved is to explore how it is lost in contexts of autoimmunity.

In this regard, the polyendocrine autoimmune disease APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) or APS-1 (autoimmune polyglandular syndrome type 1) has yielded important insights of late ([Mathis and Benoist, 2004](#)). APECED is largely monogenic, displaying autosomal recessive in-

heritance. The responsible genetic locus was identified by positional cloning as the gene encoding AIRE (autoimmune regulator), a protein with structural and functional features suggestive of a transcription factor. Mutation of the gene specifying a homologous protein in mice, termed Aire, resulted in multiorgan inflammatory infiltrates and autoantibodies (autoAbs) of diverse specificities ([Ramsey et al., 2002](#); [Anderson et al., 2002](#)). Exploration of these mutant mice as a model of APECED revealed that Aire exerts its antiautoimmunity function primarily within thymic epithelial cells and that it does this by inducing the transcription of a large set of genes encoding proteins considered to be tissue specific ([Anderson et al., 2002](#)). A series of prior studies had demonstrated such transcripts in both murine and human thymic stroma, in particular in medullary epithelial cells (MECs), and had implicated them in the establishment of T cell tolerance ([Kyewski et al., 2002](#)).

A critical next level of investigation centers on the cellular mechanism by which MECs expressing Aire promote the purging or inactivation of autoreactive specificities from the T cell repertoire. One possibility is via negative selection: Aire would somehow enhance the clonal deletion of self-reactive thymocytes. Evidence in favor of an Aire influence on negative selection was recently provided for a single TCR transgenic (tg) system ([Liston et al., 2003, 2004](#)). Another possibility invokes positive selection: Aire would promote clonal diversion of thymocytes into a regulatory T ( $T_{reg}$ ) cell phenotype. Analogous to previous observations in certain TCR tg systems ([Jordan et al., 2001](#)), should differentiating T cells recognize the MHC:self-peptide complexes displayed on MECs with the appropriate strength, they would not die but, instead, would be seduced into a distinct lineage, the population of  $CD4^{+}25^{+}T_{reg}$  cells. These cells would exit to the periphery, where they would keep self-reactive effector T ( $T_{eff}$ ) cells in check. An Aire effect on positive selection has not so far been rigorously tested. Here, we report on experiments aimed at critically evaluating both possibilities.

## Results

### No Apparent Aire Influences on $CD4^{+}25^{+}T_{reg}$ Cells

$CD4^{+}25^{+}$  T lymphocytes are the population of  $T_{reg}$  cells most profoundly characterized to date, demonstrated to exert an important influence on a diversity of immune and autoimmune responses ([Sakaguchi, 2004](#)). These cells are produced within the thymus, and can also be generated in the periphery subsequent to immune stimulation. Since it has been reported that the production of  $CD4^{+}25^{+}$  cells can be induced by expression of cognate ligand within thymic epithelial cells ([Jordan et al., 2001](#)), it seemed highly relevant to specifically assay their presence and function in mice lacking Aire.

The numbers and percentages of  $CD4^{+}25^{+}69^{-}$ , presumably  $T_{reg}$ , cells were normal in the spleen, lymph nodes and thymus of mice carrying an Aire-null mutation ([Anderson et al., 2002](#)). This observation on stand-

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ard Aire-deficient animals was recently confirmed (Kuroda et al., 2005), and, similarly, numbers of CD4<sup>+</sup>25<sup>+</sup> T cells were the same in Aire-positive and -negative hen egg lysozyme (HEL)-specific TCR tg mice (Liston et al., 2003). Given the well-known difficulties with adequately distinguishing regulatory from activated T lymphocytes purely on the basis of cell-surface markers (Sakaguchi, 2004), and the fact that mice devoid of Aire exhibit multiorgan autoimmunity, we sought an alternative approach for evaluating the presence of T<sub>reg</sub> cells. Foxp3 was recently designated a “master regulator” of the CD4<sup>+</sup>25<sup>+</sup> T<sub>reg</sub> phenotype (Fontenot et al., 2003; Hori et al., 2003); so we compared levels of this transcription factor in splenocytes and thymocytes of mice expressing Aire or not. Levels of Foxp3 transcripts were the same in the two types of animals (Figure 1A, top panel), as also recently reported (Kuroda et al., 2005). In addition, the numbers of cells expressing Foxp3 protein in the lymphoid organs of mutant and wild-type animals were very similar—the slightly fewer numbers in thymi of the former did not extend to the peripheral organs (Figure 1A, bottom panel, and Figure 1B). The similarity in representation of Foxp3-expressing cells was true both for the classical CD25<sup>+</sup> T<sub>reg</sub> population and the recently documented population of Foxp3<sup>+</sup>CD25<sup>-</sup> T cells (Fontenot et al., 2005; Figure 1B). Nonetheless, it remained an open question whether the T<sub>reg</sub> cells from mice lacking Aire could function normally.

The functional capacities of CD4<sup>+</sup>25<sup>+</sup> T lymphocytes from Aire-null mice were probed by a variety of assays. As illustrated in Figure 1C, CD4<sup>+</sup>25<sup>-</sup> T cells purified from Aire-positive and Aire-negative mice proliferated equally well when stimulated in vitro with an anti-CD3 mAb in the presence of irradiated splenocytes; in contrast, the CD4<sup>+</sup>25<sup>+</sup> T cell population taken from neither type of animal proliferated under these conditions. Addition of CD4<sup>+</sup>25<sup>+</sup> cells to the CD4<sup>+</sup>25<sup>-</sup> cultures inhibited proliferation in a dose-dependent manner, and this classic manifestation of T<sub>reg</sub> function did not depend on whether or not either the responding or the added cells expressed Aire.

To assay the function of T<sub>reg</sub> cells in vivo, we tested their ability to inhibit autoimmune pathology upon co-transfer with T<sub>eff</sub> cells into lymphopenic mice. As anticipated (Sakaguchi et al., 1995), purified CD4<sup>+</sup>25<sup>-</sup> cells induced wasting (Figure 1D) and histologically apparent colitis (Figure 1E) after transfer into *Recombination Activating Gene* (RAG)-deficient recipients, whether or not the donor from which they were derived expressed Aire. The CD4<sup>+</sup>25<sup>+</sup> population from either Aire-positive or -negative donors blocked these disease manifestations, and with the same efficiency (Figures 1D and 1E).

#### Aire Influences on Regulatory T Cells, More Generally

Although CD4<sup>+</sup>25<sup>+</sup> cells constitute a critical population of regulatory T lymphocytes, there are other T cell subsets that can exert a crucial influence on the unfolding of an immune response or development of an autoimmune disease: the Th2, Th3, Tr1, CD8<sup>+</sup>, NKT, DX5<sup>+</sup> and  $\gamma\delta$  subsets have all been cited in this regard (Jonuleit and Schmitt, 2003). It is conceivable that Aire-null mice develop multiorgan autoimmunity because of a deficiency in one or more of these T<sub>reg</sub> populations.

To test the functionality of regulatory T cells more generally, we performed a double-thymus transplant experiment. Grafting Aire-negative, but not Aire-positive, 2-deoxyguanosine (2-DG)-resistant thymic stroma into athymic nude mice led to T and B cell autoimmunity like that of Aire-null mice (Anderson et al., 2002). It is also known from studies on a variety of systems that thymic stroma, in particular thymic epithelial cells, can impose tolerance dominantly, through positive selection of T<sub>reg</sub> cells (Modigliani et al., 1995). Therefore, it might be anticipated that, if the autoimmunity that develops in mice provided with thymic stroma lacking Aire reflects its inability to mediate positive selection of T<sub>reg</sub> cells, then provision, in addition, of stroma expressing Aire should permit selection of such cells and thereby protect the mice from autoimmune disease. By twelve weeks after their transfer into nude mice, 2-DG-resistant stroma derived from either an Aire-negative or an Aire-positive thymic lobe had given rise to a functional organ, containing approximately equal numbers of thymocytes (Figure 2A) of comparable CD4/CD8 subset distribution (Figure 2B). Double-thymus recipients exhibited the same range of inflammatory infiltrates and autoAbs as those characteristic of mice hosting a single Aire-null thymus (Figure 2C), in agreement with the more limited analysis recently reported (Kuroda et al., 2005).

A second more general approach to revealing a deficit in regulatory T cells is via splenocyte cotransfer experiments. Introduction of spleen cells from Aire-negative mice into RAG-deficient animals induced multiorgan infiltrates 12 weeks later (Anderson et al., 2002). Cotransfer of an equal number of spleen cells from Aire-positive mice had no evident effect on these autoimmune manifestations (Figure 2D).

Thus, according to results from two very different assays, adding regulatory T cells produced in an Aire-expressing thymus at a 1:1 ratio to effector T cells produced in an Aire-deficient environment afforded no protection from autoimmunity. These observations argue that Aire-null mice do not develop multiorgan autoimmune disease simply because they lack regulatory T cell activity. That being said, we could suppress the appearance of inflammatory infiltrates by introducing a frank excess of T lymphocytes from normal donors—either by cotransplanting stroma from one mutant plus four wild-type thymic lobes into athymic recipients, or by cotransferring Aire-negative plus four times the number of Aire-positive splenocytes into RAG-deficient hosts (Figures 2C and 2D, lower right panels). However, it is difficult to evaluate the significance of these observations for a number of reasons, including the fact that we do not know which cell type(s) is (are) effecting the inhibition. We favor the explanation that the dampening merely represents a nonspecific crowding effect (Barthlott et al., 2003).

#### Aire Controls the Emergence of Self-Reactive Effector T Cells

We then shifted our attention to the possibility that Aire controls autoimmunity by promoting negative selection, i.e., clonal deletion of self-reactive thymocytes. TCR tg systems of diverse types were examined, cover-

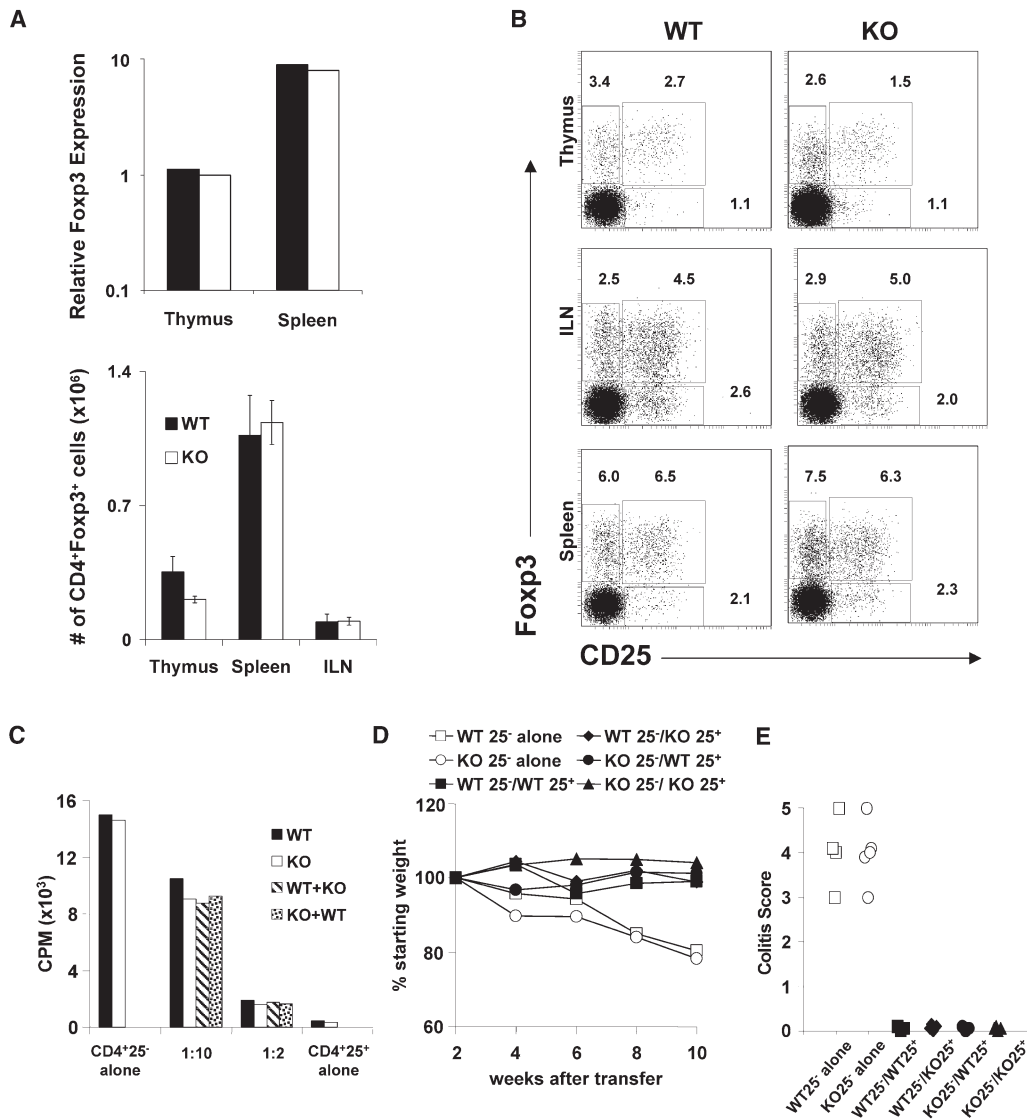


Figure 1. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> Cells in Aire-Deficient Mice Can Efficiently Suppress T<sub>eff</sub> In Vitro and In Vivo

(A) Top, RT-PCR analysis of Foxp3 (relative to HPRT) expression levels from Aire-deficient and wild-type mice. Representative of three independent experiments with one to two mice each. Bottom, number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells per organ. ILN, inguinal lymph node (n = 3). The data represent the average ± SD for each group.

(B) Flow cytometric analysis of intracellular Foxp3 expression in CD4<sup>+</sup>CD8<sup>-</sup> cells, determined using the anti-Foxp3 mAb FJK-16s. Numbers are the percentages of CD4<sup>+</sup>CD8<sup>-</sup> cells in each marked region.

(C) Purified populations of CD4<sup>+</sup>CD25<sup>-</sup> T<sub>eff</sub> alone (5 × 10<sup>4</sup> cells), CD4<sup>+</sup>25<sup>+</sup> T<sub>reg</sub> cells alone (2.5 × 10<sup>4</sup> cells), or the two purified populations were mixed as indicated at the following ratios of T<sub>reg</sub>:T<sub>eff</sub> (1:10 = 5 × 10<sup>3</sup> : 5 × 10<sup>4</sup> cells; 1:2 = 2.5 × 10<sup>4</sup> : 5 × 10<sup>4</sup> cells) from Aire-deficient or wild-type mice, and all were assayed for proliferation in the presence of irradiated B6 splenocytes and anti-CD3.

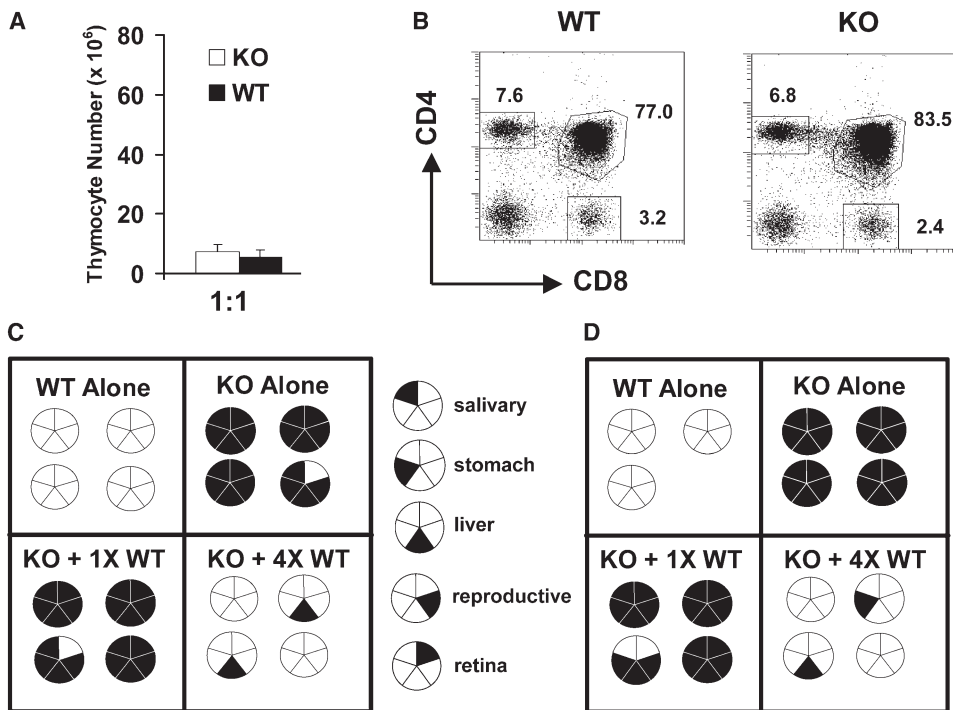
(D) 4 × 10<sup>5</sup> purified CD4<sup>+</sup>CD25<sup>-</sup> T<sub>eff</sub> cells were transferred i.v. into RAG-deficient hosts with or without 1 × 10<sup>5</sup> purified CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in a criss-cross fashion from Aire-deficient or wild-type donors. Recipients were weighed weekly to monitor the incidence of colitis, and were followed for 12 weeks.

(E) At the end of 12 weeks, colon sections were prepared, stained, and scored for the severity of colitis.

ing antigens expressed in different thymic stromal cell types, antigens located in various subcellular compartments, and MHC class II-restricted, CD4<sup>+</sup> versus class I-restricted, CD8<sup>+</sup> T cell specificities.

HY TCR tg mice carry rearranged transgenes that encode a TCR capable of recognizing a peptide from the male-specific antigen, HY (now known to correspond to Smcy), in the context of D<sup>b</sup> (Markiewicz et al., 1998). In females, on the standard C57Bl/6 (B6) genetic back-

ground, HY is not synthesized; in males, it is widely expressed (Xu et al., 2002), including within the hematopoietic and epithelial cell compartments of the thymic stroma. As a consequence, female HY TCR tg mice have an overabundance of CD8<sup>+</sup> T cells displaying the HY specificity (Kisielow et al., 1988b; Figure S1A, left-most panel, in the Supplemental Data available with this article online), while male transgenics have few mature T lymphocytes, whether CD8<sup>+</sup> or CD4<sup>+</sup>; indeed,



**Figure 2.** Cotransfer of Equal Numbers of Aire-Deficient and Wild-Type Thymic Stroma or Mature Splenocytes Does Not Inhibit Autoimmunity  
Athymic B6 nude mice were transplanted with either one thymus lobe from Aire knockout or wild-type donors, one lobe each from an Aire knockout and wild-type donor, or one lobe from an Aire knockout donor and four from wild-type donors. Recipient mice were aged for 10–12 weeks and then analyzed for thymic reconstitution by counting total cell number and by flow cytometry (yields are from single thymic grafts in recipient mice) (A and B), and for autoimmune infiltrates by H&E, scored as shown (C). B6.RAG mice were used as recipients for transfer of wild-type or knockout splenocytes in the various numbers shown. Recipient mice were aged for 10–12 weeks, and were then analyzed for autoimmune infiltrates by H&E and scored as shown (D). Immune reconstitution of splenic transfers was confirmed by total cell counts and flow cytometric analysis of the spleen for CD4, CD8, and B220 markers (data not shown).

there are few thymocytes beyond the CD4<sup>-</sup>CD8<sup>-</sup> (double-negative, DN) stage (Kisielow et al., 1988a; Figure S1A, right-center panel). Introduction of an Aire-null mutation into the HY TCR tg system had no substantial effect on thymocyte numbers, (Figure S1B), thymocyte positive selection in females or negative selection in males (Figure S1A, left-center and right-most panels). This is the expected result given the early deletion of HY thymocytes, at the DP stage, reflecting widespread expression of the relevant antigen in stromal cells, including both Aire-positive and -negative cell types in wild-type mice.

When propagated on the standard B6 genetic background, DEP TCR tg mice have an overabundance of CD4<sup>+</sup> T cells that recognize a peptide of human c-reactive protein (hCRP) presented by A<sup>b</sup> molecules (Klein et al., 1998; Figure S2A, left-most panels). When the DEP mouse line is crossed with a second tg line harboring an hCRP transgene driven by its own promoter/enhancer elements, few mature CD4<sup>+</sup> T cells emerge because of clonal deletion of CD4<sup>+</sup>8<sup>-</sup> and a substantial fraction of CD4<sup>+</sup>8<sup>+</sup> (double-positive, DP) thymocytes (Klein et al., 1998; Figure S2A, right-center panels). The double-transgenics expressed hCRP, a soluble molecule, in liver cells and in thymic MECs; in females (with low hCRP levels), negative selection of DEP thymocytes was found to depend critically on the latter cell type

(Klein et al., 1998). Introduction of an Aire-null mutation into this double-tg system had little influence on negative selection of thymocytes from female DEP mice (Figures S2A and S2B). This observation fits well with our finding that the levels of CRP transcripts in thymic MECs were not detectably influenced by expression of Aire (data not shown; and Anderson et al., 2002) and the fact that CRP is a soluble molecule and thus can be picked up and presented by non-MEC APCs in the vicinity.

Since Aire controls transcript levels of only a subset of the peripheral-tissue antigen genes ectopically expressed in thymic MECs (Anderson et al., 2002), we wished to investigate a TCR tg system keyed on an antigen whose MEC expression has already been demonstrated to be tightly regulated by Aire. Preproinsulin II is such an antigen (Anderson et al., 2002), prompting us to choose an insulin-based double (TCR/antigen) tg system. The RIP-mOVA line expresses a membrane-bound form of ovalbumin under the dictates of the rat *insulin* gene promoter, consequently in the pancreatic islets and thymus (also, aberrantly, in the kidneys and testis) (Kurts et al., 1996). OT-II is a TCR tg line carrying rearranged transgenes encoding a TCR that recognizes an ovalbumin peptide in the context of A<sup>b</sup>, and thereby has a surfeit of ovalbumin-reactive CD4<sup>+</sup> T cells when propagated on the standard B6 genetic background

(Barnden et al., 1994; Figures 3A and 3B, left-most columns). Introduction of cognate antigen by generation of RIP-mOVA/OT-II double-tg mice leads to clonotype deletion within the thymus, evidenced by a reduction in numbers of total and of CD4<sup>+</sup>8<sup>-</sup> thymocytes (Figure 3C, left and left-center panels), alterations in CD4/CD8 thymocyte and splenocyte subset distributions (Figures 3A and 3B, left-center columns) and loss of clonotype (V<sub>β</sub>5<sup>+</sup>V<sub>α</sub>2<sup>+</sup>)-positive CD4<sup>+</sup> cells from the thymus and spleen (Figures 3A and 3B, left-center columns; Figure 3C, right-center and right panels). Introducing the Aire-null mutation into double-tg mice did not noticeably affect these parameters when in the heterozygous state (Figures 3A and 3B, right-center columns, and Figure 3C). However, in the homozygous state, the *Aire* gene mutation had profound consequences, strongly inhibiting negative selection of the autoreactive clonotype (Figures 3A and 3B, right-most columns, and Figure 3C). Indeed, most parameters substantially, though usually not completely, reverted to the single-tg, OT-II-transgene-only, values.

The absence of Aire did not lead to a reduced generation of clonotype-positive CD4<sup>+</sup>25<sup>+</sup>, presumably T<sub>reg</sub>, cells. While the percentage of T cells with this phenotype was lower in Aire-negative than in Aire-positive double-tg thymi (Figure 4, bottom left), this was accompanied by a reduction in clonotype deletion (Figure 3A, bottom row), such that the number of these cells remained fairly constant (Figure 4, bottom right). Overall, the preferential resistance of CD4<sup>+</sup>25<sup>+</sup>, versus CD4<sup>+</sup>25<sup>-</sup>, cells to negative selection, rather than their increased positive selection, when cognate ligand is expressed in thymic epithelial cells parallels recent findings on a different double-tg system (van Santen et al., 2004), and independently argues that Aire does not exert its primary influence on the positive selection of regulatory T cells.

#### Aire Control of Autoimmune Disease

In order to extend our conclusions to an MHC class-I-restricted system entailing CD8<sup>+</sup> T cells, we paired the RIP-mOVA line with the OT-I TCR tg line. The latter has a T cell repertoire highly enriched for a CD8<sup>+</sup> clone recognizing the SIINFEKL peptide of ovalbumin presented in the context of K<sup>b</sup>. In an Aire wild-type context, double-tg mice showed a clear deletion phenotype, evidenced by a reduction in the fraction of clonotype-positive CD8<sup>+</sup> T cells in the thymus *vis à vis* the corresponding values in single-tg OT-I-only animals (Figure 5A and left panel of Figure 5C) and clonotype-positive cells in the spleen (Figure 5B and center and right panels of Figure 5C). These fractions were substantially increased, although not to wild-type levels, in double-tg animals in an Aire-mutant context. In addition, the mature CD8<sup>+</sup> cells that did emerge in *Aire*<sup>-/-</sup> mice appeared to express reduced levels of the clonotypic TCR (cf rightmost and leftmost panels in Figure 5A.)

The RIP-mOVA/OT-I double-tg mice maintained in our animal facility do not develop diabetes (Figure 5D). Strikingly, all homozygous Aire-null double-tg animals developed diabetes at birth; and, surprisingly, heterozygous mutant double transgenics also become diabetic, though onset was delayed until after 5 days of age. Nei-

ther RIP-mOVA nor OT-I single-tg mice on an Aire-deficient background showed any signs of diabetes (data not shown). Tissue sections from homozygous Aire-null, but not from wild-type, RIP-mOVA/OT-I animals (Figure 5E) revealed an aggressive lymphocytic infiltrate in the pancreas. All islet tissue had been destroyed. Aire-null double-tg heterozygotes also showed pancreas infiltrates (data not shown). (It may be worth noting that the diseased state of the Aire-mutant mice, in particular of the homozygotes, depicted in Figure 5 probably accounts for the atypical appearance of their thymocyte profiles. Resulting stress leading to steroid hormone production and eventually to apoptosis of immature thymocytes may also explain why in this case rescue of clonotype-positive cells in *Aire*<sup>-/-</sup> OVA<sup>+</sup> mice did not “rebound” to the levels in their *Aire*<sup>-/-</sup> OVA<sup>-</sup> counterparts.)

#### Something Else?

The choice of the RIP-mOVA/OT-I,II systems for these studies was based on the fact that transcription of the endogenous *ins2* gene in thymic MECs is tightly controlled by Aire (Anderson et al., 2002). We assumed that synthesis of mOVA transcripts driven by the rat insulin promoter in a tg context would be regulated in parallel, especially since the pattern of expression of SV40 T antigen transcripts driven by the very same promoter provided the first evidence of ectopic thymic expression of peripheral-tissue antigen genes (Jolicœur et al., 1994). Nevertheless, we felt it important to experimentally verify this assumption. To that end, we quantitated expression of mOVA transcripts in thymi of 3- to 6-week-old wild-type, Aire-null heterozygous and Aire-null homozygous RIP-mOVA tg mice by an RT-PCR assay. Entirely unexpectedly, the three types of animals exhibited essentially the same level of thymic mOVA transcripts (Figure 6A). Thinking that our assay might not be sufficiently discriminating, we purified thymic MECs from Aire-positive and -negative RIP-mOVA mice and assayed expression of the mOVA gene. Again, levels of mOVA transcripts were indistinguishable for these two types of animals (Figure 6B). In contrast, transcripts derived from the endogenous *ins2* gene were expressed at a level four orders of magnitude higher in thymic MECs from Aire-positive mice (Figure 6C). We cannot but conclude that, in MECs of RIP-mOVA tg mice, Aire does not regulate rat insulin promoter-driven synthesis of mOVA transcripts.

Yet, as was clear from the data presented in Figures 3 and 4, Aire does control clonal deletion of self-reactive thymocytes. Therefore, it must be exerting influences on processes in addition to the transcription of peripheral-tissue antigens. To obtain insight into what these other processes might be, we returned to our gene expression databases on thymic Aire-positive and -negative MECs (Anderson et al., 2002), reanalyzed recently using more current bioinformatics tools (Johnnidis et al., 2005). This time we focused on those genes positively regulated by Aire that did not encode peripheral-tissue antigens as well as genes negatively regulated by Aire. Intriguingly, many of the loci so highlighted encode proteins involved in antigen processing or presentation, e.g., *li*, *H2-M*, *H2-O*, *Tap1*, *ctsL*, *mec11*, *gilt*,

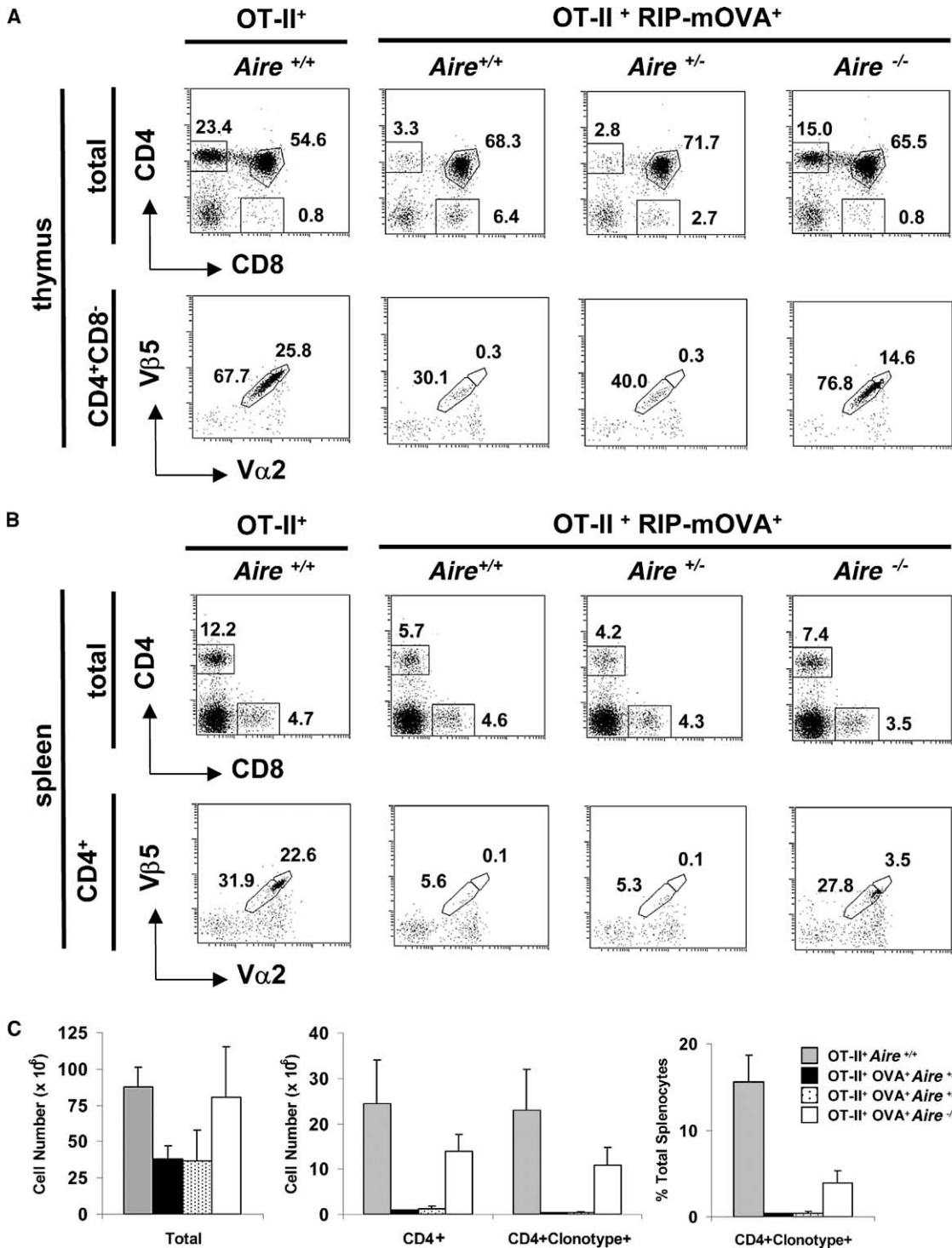


Figure 3. Aire Regulates Negative Selection of the OT-II<sup>+</sup>RIP-mOVA<sup>+</sup> tg Line

OT-II TCR tg and OT-II<sup>+</sup>RIP-mOVA<sup>+</sup> double-tg mice were produced as *Aire*<sup>+/+</sup> (n = 3 and 3), *Aire*<sup>+/-</sup> (n = 3 and 7), or *Aire*<sup>-/-</sup> (n = 3 and 5). Thymocytes and splenocytes from 5- to 6-week-old mice were counted and stained with anti-CD4, -CD8, and the anticlonotypic combination of anti-Vα2 TCR and anti-Vβ5 TCR mAbs.

(A) Representative cytofluorimetric plots of CD4 versus CD8 staining of total thymocytes from individual mice are shown in the upper row. In the second row are plots for Vα2 and Vβ5 staining, gated on CD4<sup>+</sup>CD8<sup>-</sup> cells.

(B) shows CD4 versus CD8 staining of total splenocytes in the first row, and Vα2 and Vβ5 staining of CD4<sup>+</sup> splenocytes in the second row.

(C) Average total thymocyte, CD4-SP thymocyte, and CD4<sup>+</sup>CD8<sup>-</sup> clonotype<sup>+</sup> thymocyte numbers, as ascertained by counting on a hemacytometer and cytofluorimetric analysis. The data represent the average ± SD for each group.

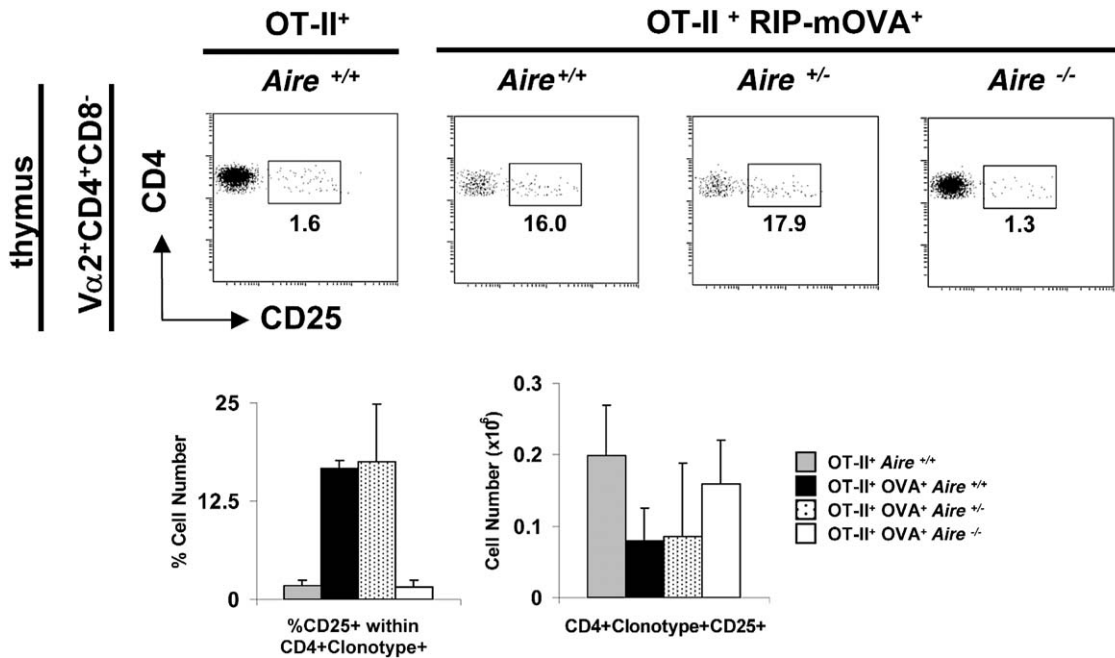


Figure 4. Aire Does Not Regulate Positive Selection of CD4<sup>+</sup>CD25<sup>+</sup> Cells of the OT-II<sup>+</sup>RIP-mOVA<sup>+</sup> tg Line

The same thymocytes analyzed in Figure 3A were also stained with anti-CD25 mAb. Shown is CD4 versus CD25 staining on V $\alpha$ 2<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> thymocytes (top); average % CD25<sup>+</sup> cells within CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>CD8<sup>-</sup> thymocytes (bottom left); and total number of CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup>CD25<sup>+</sup>CD8<sup>-</sup> thymocytes (bottom right). The data represent the average  $\pm$  SD for each group.

*erp57*, and *bip* (Figure 7A). Several others specified chemokines (*ccl17*, *ccl22*, *cxcl9*, *ccl19*, *cxcl10*, and *ccl25*), certain of which are known to be involved in thymocyte trafficking to or within the medulla (Kwan and Killeen, 2004). Most of these chemokine gene expression changes have already been confirmed by RT-PCR (data not shown). Still other loci specified cytokines (*IL-9*, *IL-12a*, and *IL-4*).

The alterations in expression of several genes encoding molecules engaged in the MHC Class I and Class II antigen processing/presentation pathways suggested that Aire might somehow affect antigen presentation by thymic MECs. We tested this possibility in two types of *in vitro* assay. The first entailed presentation of endogenously synthesized protein: splenocytes from OT-I/RAG<sup>o/o</sup> mice were challenged with MECs purified from either Aire wild-type or Aire knockout RIP-mOVA mice. MECs that did not express Aire were less effective antigen presenters (Figure 7B), and this difference was observed over a range MEC:OT-I ratios (Figure 7C). The second assay involved presentation of exogenously added peptide: OT-I/RAG<sup>o/o</sup> splenocytes challenged with wild-type or knockout MECs plus SIINFEKL peptide. Again, Aire-negative MECs were less competent (Figure 7D) over a range of APC:T cell ratios (Figure 7E).

One simple explanation for the diminished capacity of Aire<sup>-/-</sup> MECs to present antigens would be that they express lower levels of MHC molecules. As illustrated in Figure 7F, this is not the case, neither for the MHC Class II<sup>lo</sup> nor the MHC Class II<sup>hi</sup> subsets, the latter, which also display greater numbers of costimulatory molecules, presumably most apt at antigen presenta-

tion. There were also no substantial differences in the levels of the costimulatory molecules CD80, CD86, PD-1, PD-L1, or PD-L2 expressed by Aire-positive and -negative MECs (data not shown).

Lastly, in contrast to what was seen with MECs, purified CD11c<sup>+</sup> splenic DCs from Aire-null mice were as effective as those from Aire wild-type littermates at presenting the SIINFEKL peptide to cultured OT-I T cells (if not more so) (Figure 7G).

## Discussion

The original description of Aire gene knockout mice—in particular, their dearth of thymic peripheral-tissue transcripts coupled with their multiorgan autoimmunity—served to rehighlight the importance of central mechanisms of immunological tolerance (Anderson et al., 2002). Subsequently, evidence was provided that Aire exerts its influence on the central induction of tolerance by regulating the clonal deletion of self-reactive thymocytes (Liston et al., 2003, 2004). Our present report offers three important observations: (1) Aire affects central tolerance primarily by controlling the negative selection of T<sub>eff</sub> cells, and not the positive selection of T<sub>reg</sub> cells; (2) Aire must impinge on clonal deletion at a point (or points) in addition to its regulation of the expression of peripheral-tissue transcripts in thymic MECs; and (3) Aire function is critical for keeping overt autoimmune disease in check.

### Negative versus Positive Selection

In theory, Aire could influence the imposition of central tolerance via either (or both) of two cellular mechanisms:

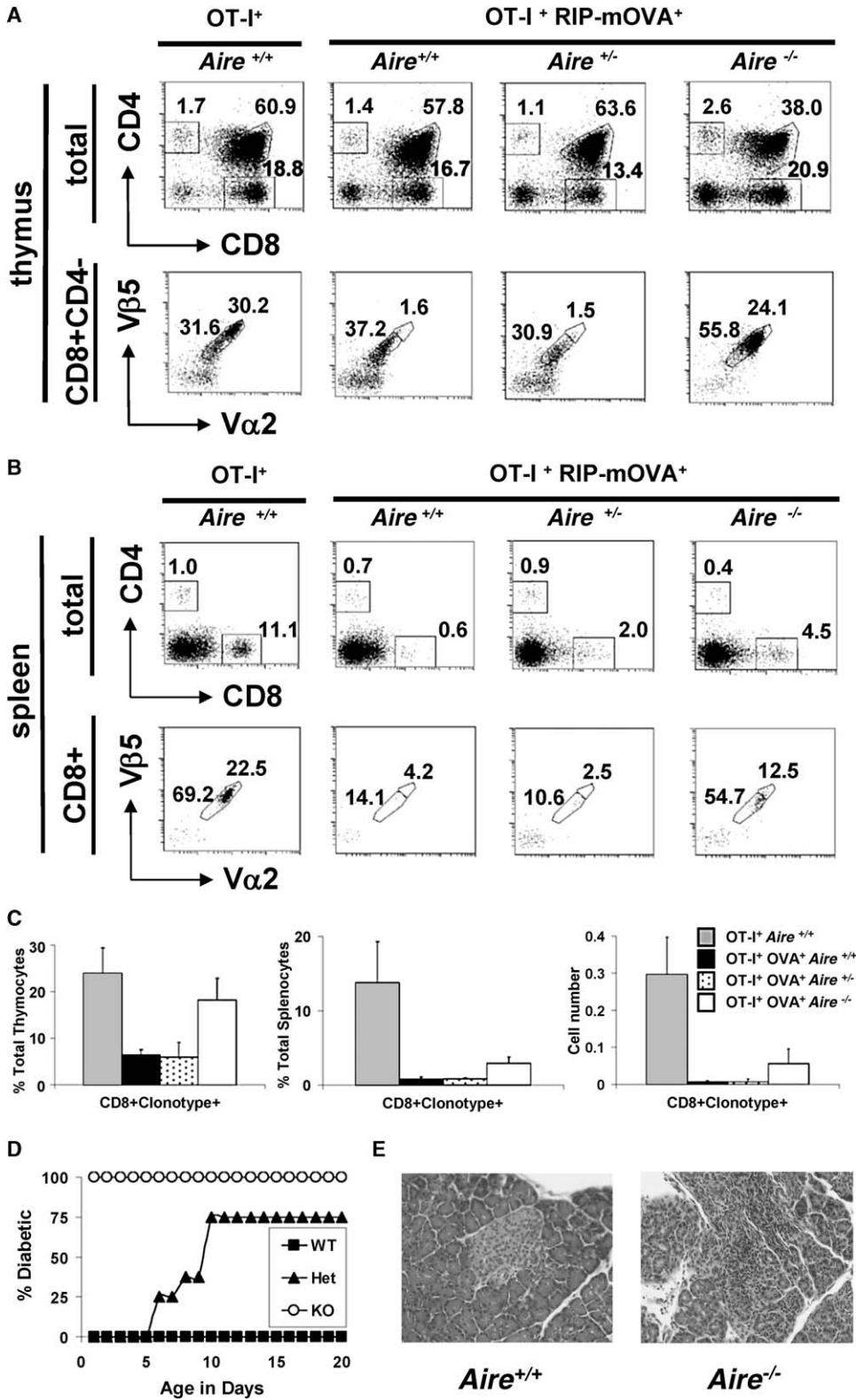
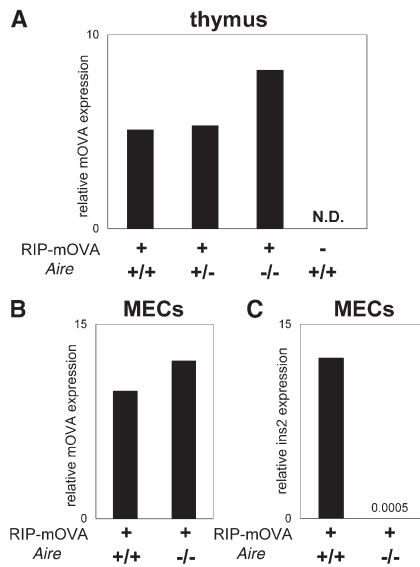


Figure 5. Aire Regulates Negative Selection and Prevents Diabetes in OT-I<sup>+</sup>RIP-mOVA<sup>+</sup> Mice

OT-I tg and OT-I<sup>+</sup>RIP-mOVA<sup>+</sup> tg mice were produced as *Aire*<sup>+/+</sup>, (n = 4 and 5), *Aire*<sup>+/-</sup> (n = 4 and 4) or *Aire*<sup>-/-</sup> (n = 4 and 5). Splenocytes and thymocytes were prepared from newborn mice, counted and stained with anti-CD4, anti-CD8, and the anticolonotypic combination of anti-Vα2 TCR and anti-Vβ5 TCR mAbs. [Newborn mice were analyzed because Aire-deficient OT-I<sup>+</sup>RIP-mOVA<sup>+</sup> mice developed diabetes at birth (see below) and died in the first few days of life.]





**Figure 6. mOVA Transcripts Are Present in *Aire*<sup>-/-</sup> Thymic MECs**  
Relative expression of mOVA (A) and (B) or insulin2 (C) was determined by quantitative real-time PCR (TaqMan) on cDNA prepared from whole thymus (A) or cytofluorimetrically-sorted (CD45<sup>-</sup>, G8.8<sup>+</sup>, CDR1/Ly51<sup>int</sup>) MECs (B) and (C). Expression values are shown in arbitrary units, normalized relative to HPRT. Data shown in (A) and (B) are representative of two independent experiments.

by enhancing the clonal deletion of self-reactive thymocytes, or by reducing the clonal diversion of thymocytes into a regulatory T cell phenotype. Predictions as to the phenotype of Aire-deficient mice are simple, then: in the first instance, escape into the periphery of self-reactive T<sub>eff</sub> cells that failed to encounter cognate ligand in the thymus; in the second, an impoverishment in the periphery of T<sub>reg</sub> populations, in particular CD4<sup>+</sup>25<sup>+</sup>69<sup>-</sup> cells.

While an effect on clonal deletion of T cells seems *a priori* the more straightforward explanation, this scenario has been challenged by a certain number of investigators, primarily because of the potentially very small number of thymic cells expressing peripheral-tissue transcripts. MECs are rather rare, constituting only a few percent of thymic stromal cells (reviewed in Boyd et al., 1993); worse, it has been suggested that at any given time any given peripheral-tissue transcript may be expressed by only a fraction (as low as 0.5%) of thymic MECs (Derbinski et al., 2001). It was felt that so few cells would not be able to effectively purge the T cell repertoire of self-reactive specificities, but should be capable of selecting enough regulatory cells to keep them in check.

Therefore, we considered it imperative to directly test Aire's influence on the positive selection of T<sub>reg</sub> cells — which had not been evaluated before, either in the original descriptions of the Aire-null mice (Ramsey et al., 2002; Anderson et al., 2002) or in the subsequent reports from Liston et al (Liston et al., 2003, 2004).

Our data argue that Aire does not operate primarily through an influence on the positive selection of T<sub>reg</sub> cells. CD4<sup>+</sup>25<sup>+</sup>69<sup>-</sup> cells were present in normal numbers in Aire-deficient mice; and they exhibited the usual Foxp3 transcript and protein levels, suppressive effect in vitro, and in vivo regulatory activities in a number of assays. Perhaps most revealing was the observation that cotransfer of Aire-positive thymic stroma was not able to overcome the autoimmunity resulting from transfer of Aire-negative stroma into a thymusless recipient because this experiment assays for all regulatory cell populations, not just the now-classical CD4<sup>+</sup>25<sup>+</sup> T<sub>reg</sub> cells. The one caveat to this conclusion is that, at a ratio of 4:1, cells from the thymus or spleen of wild-type mice could dampen the autoimmunity promoted by an Aire deficiency. At present, we have no idea what type(s) of cells is (are) responsible for this suppression (and it seems unwise to launch an effort to identify them given the exaggerated nature of this assay). Nor do we know to what extent this inhibitory activity reflects an influence of competitive or of homeostatic mechanisms. We favor a “space” explanation due to well-described precedents (Barthlott et al., 2003).

*A posteriori*, it seems that the argument for an effect on positive selection based on too few Aire-expressing cells to accomplish negative selection was misplaced. First of all, mature single-positive thymocytes spend two weeks in the medulla before exiting to the periphery—so they have the time to encounter rare cells and undergo deletion (Rooke et al., 1997). Second, real-time imaging of thymus cultures has revealed that the DP thymocytes migrate extensively, in random trajectories—so they have the occasion (Witt et al., 2005). Lastly, it was demonstrated some years ago that as few as 100 APCs suffice to purge autoreactive specificities from the emerging T cell repertoire (Matzinger and Guerder, 1989; Merkschlagler et al., 1997).

On the other hand, our data add significant support in favor of the notion that Aire functions via an effect on the negative selection of T<sub>eff</sub> cells. As expected, given that thymic stromal cells in addition to MECs express their cognate antigens, or that they recognize a soluble antigen (Klein et al., 1998) whose synthesis is probably not Aire-regulated (Anderson et al., 2002), respectively, HY and DEP thymocytes were clonally deleted equally well in the presence or absence of Aire. This finding is also consistent with their early deletion before the SP stage (Figures

- (A) Representative flow cytometric plots of the lymphocyte gate for thymocytes. CD4 versus CD8 staining for individual mice in the upper row, and Vβ5 versus Vα2 staining, gated on CD4<sup>+</sup>CD8<sup>+</sup> cells, in the second row. (Thymus plots are atypical because the mice are newborn, and because the diabetes in the Aire-deficient OT-I\*<sup>RIP-mOVA</sup> mice greatly altered the staining profile of immature DP and DN populations.)  
 (B) Representative flow cytometric plots of the lymphocyte gate for splenocytes. CD4 versus CD8 staining for individual mice in the upper row, and Vβ5 vs Vα2 staining, gated on CD4<sup>+</sup>CD8<sup>+</sup> cells, in the second row.  
 (C) Average percent CD4<sup>+</sup>CD8<sup>+</sup>clonotype<sup>+</sup> thymocytes and splenocytes for the indicated mice in each group and the total number of CD8<sup>+</sup>clonotype<sup>+</sup> splenocytes. The data represent the average ± SD for each group.  
 (D) Diabetes incidence curves for OT-I\*<sup>RIP-mOVA</sup> mice that were *Aire*<sup>+/+</sup> (n = 5), *Aire*<sup>+/-</sup> (n = 8) or *Aire*<sup>-/-</sup> (n = 6). Diabetes was scored as reflecting both a positive urine dipstick test and a serum glucose concentration >250 mg/dl.  
 (E) Representative H&E staining of pancreas sections from an OT-I\*<sup>RIP-mOVA</sup> *Aire*<sup>+/+</sup> (left) and *Aire*<sup>-/-</sup> (right) newborn mouse.

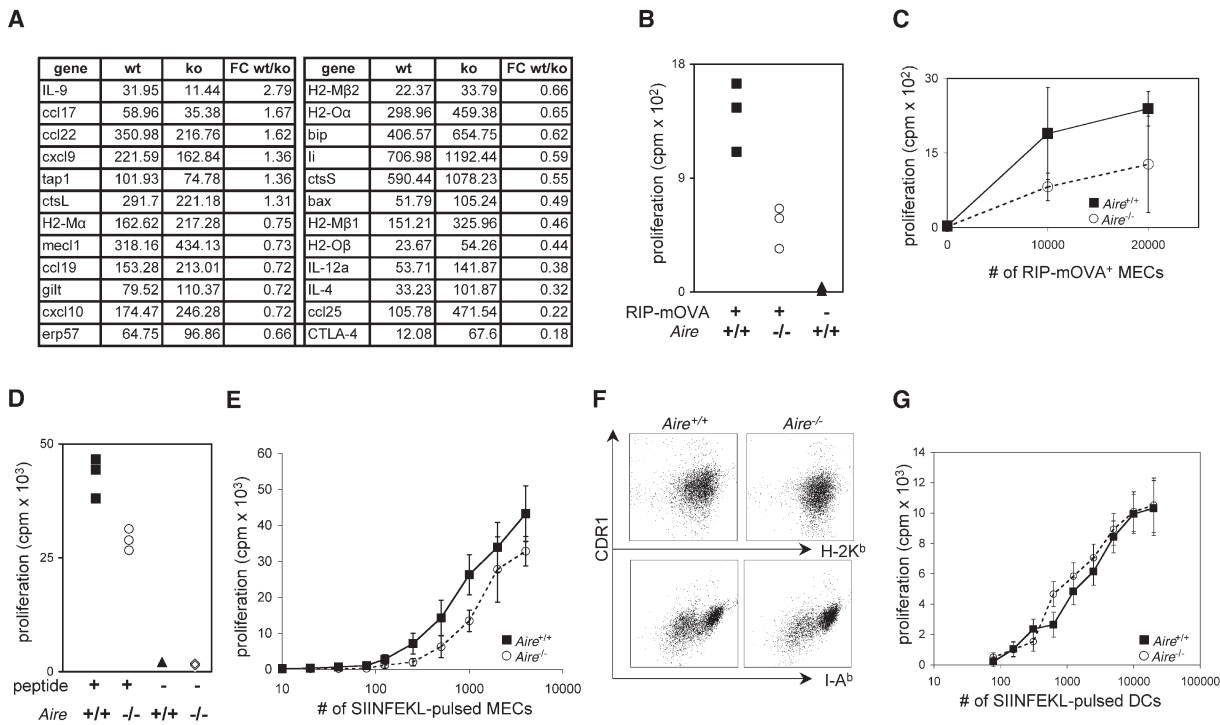


Figure 7. Inefficient Antigen Presentation by *Aire*<sup>-/-</sup> MECs

(A) List of genes whose expression levels differ between *Aire*<sup>+/+</sup> (wt) and *Aire*<sup>-/-</sup> (ko) MECs, according to the Affymetrix gene-chip analysis originally presented in (Anderson et al., 2002). FC, fold change. Genes shown were selected because of their known or possible roles in antigen processing, presentation, or MEC-thymocyte interaction. In (B–E), sorted thymic MECs from 3- to 6-week-old mice were used as APCs to stimulate OT-I\**RAG*<sup>-/-</sup> splenocytes in vitro. In (B), 10<sup>4</sup> RIP-mOVA transgenic MECs were combined with 5 × 10<sup>4</sup> OT-I\**RAG*<sup>-/-</sup> splenocytes. Each point represents one well. (B) is representative of four independent experiments with three to six replicate wells for each condition per experiment. When the results from all four experiments were combined (normalized to the mean T cell response to *Aire*<sup>+/+</sup> MECs), the difference between the proliferative responses to *Aire*<sup>+/+</sup> MECs and *Aire*<sup>-/-</sup> MECs was 2.5-fold, with a p value < 0.02. In (C), 0, 10<sup>4</sup>, or 2 × 10<sup>4</sup> RIP-mOVA transgenic MECs were combined with 5 × 10<sup>4</sup> OT-I\**RAG*<sup>-/-</sup> splenocytes. Points are the average ± SD of three to six wells per condition. In (D) and (E), nontransgenic MECs were pulsed with 0.01 mM (D) or 1 μM (E) SIINFEKL peptide, washed extensively to eliminate free peptide, and combined with 5 × 10<sup>4</sup> OT-I\**RAG*<sup>-/-</sup> splenocytes. In (D), 10<sup>4</sup> SIINFEKL-pulsed MECs were used, and each point represents one well. In (E), the number of pulsed MECs was titrated, and each point is the average ± standard deviation of 12 wells per condition. (E) is representative of four independent experiments. The data represent the average ± SD for each group. The mean reduction in proliferative response to SIINFEKL-pulsed *Aire*<sup>+/+</sup> MECs, relative to *Aire*<sup>+/+</sup> MECs in the nonsaturating section of the curve (250 to 1000 MECs) averaged 53.5% ± 28.0% over all experiments. The response of OT-I\**RAG*<sup>-/-</sup> splenocytes to unpulsed MECs or to MECs pulsed with irrelevant peptide (data not shown) was at background level. (F) demonstrates that the inefficient antigen presentation by *Aire*<sup>+/+</sup> MECs is not due to reduced surface expression of MHC class I or II molecules. The cells shown are CD45<sup>-</sup> thymic stromal cells. In (G), splenic CD11c<sup>+</sup> DCs were positively selected with anti-CD11c magnetic beads, then incubated with 1 μM SIINFEKL peptide, washed extensively to eliminate free peptide, and combined with 5 × 10<sup>4</sup> OT-I\**RAG*<sup>-/-</sup> splenocytes. The number of pulsed DCs was titrated, and each point is the average ± the SD of three wells per condition.

S1 and S2). In contrast, we anticipated that OT-I and OT-II thymocytes would not be deleted as efficiently in thymi of Aire-deficient mice given multiple reports that both the endogenous *ins2* gene and RIP-driven transgenes are expressed rather specifically in MECs. Indeed, the clonal deletion of thymocytes displaying the OT-I and OT-II TCRs was compromised, though not completely eliminated, in Aire-deficient mice (Figures 3 and 5). As already discussed, incomplete “rebound” of clonotype-positive thymocyte and T cell numbers, particularly in the case of OT-I, could reflect the diseased state of these animals, born with type-1 diabetes.

#### An Additional Function for Aire

We were surprised to observe that RIP-driven expression of OVA in thymic MECs was not, in fact, regulated by Aire.

Given that transcription of the endogenous *ins2* gene was strictly Aire dependent, the conclusion has to be that the RIP fragment does not include the sequence elements required for Aire to exert its function, whether direct or indirect, and/or that particular sites of chromosomal integration might be capable of overriding its influence. Integration-site effects on RIP-driven expression of transgenes in the thymic stroma have been described before (Smith et al., 1997) and, in contrast to our findings, Liston et al. very recently reported that Aire does control expression of their RIP-HEL construct (Liston et al., 2004), though they did not confine their examination specifically to thymic MECs. Our surprising observation does not take away from the fact that Aire was found to control the synthesis of transcripts encoding a battery of peripheral-tissue antigens in thymic MECs (Anderson et al., 2002);

nor does it negate the finding that Aire can influence the clonal deletion of T cells that recognize antigens made by MECs (Figures 3 and 5). It just means that the experiments reported here do not directly link the two — that a dearth of MEC transcripts encoding a particular protein was not directly demonstrated to result in a loss of T cell tolerance to that protein. Relatedly, it was recently shown that, while  $\alpha$ -fodrin was a target of autoimmunity in *Aire*<sup>-/-</sup> mice, transcripts of the *\alpha*-fodrin gene did not appear to be diminished in total thymic stroma of knockout versus wild-type animals (Kuroda et al., 2005).

Hence, Aire must be controlling processes other than the thymic expression of peripheral-tissue antigens. We looked for clues to the identity of such processes by examining the list of genes that were either positively regulated by Aire but did not specify peripheral-tissue antigens, or were negatively regulated by Aire. Some very suggestive genes emerged. For example, several chemokine genes were Aire regulated, mostly positively, including certain ones implicated in thymocyte activities in the medulla. It is easy to envisage how changes in chemokine gradients might alter thymocyte access or attachment to MECs. There were also changes in loci encoding a number of molecules implicated in the processing and presentation of antigens to T cells. Particularly interesting examples are H2-M and H2-O, which have been suggested to play an important role in editing the repertoire of peptides displayed by MHC Class II molecules (Brocke et al., 2002). Similarly, several molecules implicated in shaping the repertoire of MHC class I molecules [eg tap1, cathepsin L, mecl1, gilt, erp57, bip (Paulsson, 2004)] are included on the list. Aire's reported activity as an E3 ubiquitin ligase (Uchida et al., 2004) may also be relevant, although the existence of such an activity was recently challenged (Bottomley et al., 2005).

Searching for a defect in antigen presentation by thymic MECs from Aire-null mice proved immediately fruitful: their presentation of both endogenously synthesized OVA and the appropriate exogenously added OVA peptide to OT-I T cells in culture was less effective in the absence of Aire. The expression of MHC molecules and of the several costimulatory molecules tested was not reduced in the absence of Aire, and so could not explain this difference. This result is consistent with the observation that the two types of MECs elicited indistinguishable Ca<sup>++</sup> fluxes in responding thymocytes (data not shown). It is now imperative to define the mechanism responsible for this defective antigen presentation by the MECs, as well as to determine to what extent this defect is responsible for the inadequate tolerance of Aire KO mice. In the meantime, we are left with the thought that to promote tolerance it makes perfect sense to both equip MECs with an array of self-peptides and better arm them as antigen presenters.

Why, then, did clonal deletion operate normally in the HY and DEP systems? In the first case, the cognate antigen is expressed in essentially all cell types, promoting deletion at an early DP stage when thymocytes are still in the cortex, so we would not expect to see an Aire effect. In the second instance, the corresponding antigen is expressed primarily in thymic MECs, though probably not under the control of Aire (Anderson et al., 2002); but, since the transgene-encoded hCRP is synthesized in soluble form, it can be picked up and presented by other cell types in the vicinity, as has been recently described (Gal-

legos and Bevan, 2004). Here, again, clonal deletion at a premedulla DP stage was observed and, again then, we would not expect to see an Aire effect. This situation is different from the one recently reported for the OT-II system (Gallegos and Bevan, 2004), where mOVA is not secreted, but is "handled" by MECs and "handed off" to hematopoietic cells in a form and by a mechanism currently unknown. For example, it was recently shown that one possible mechanism of crosspresentation is intercellular peptide transfer through gap junctions (Neijssen et al., 2005).

### Keeping Autoimmune Disease in Check

While Aire-deficient mice on the mixed B6x129 genetic background exhibited multiple manifestations of autoimmunity, this immunological overreaction generally fell short of overt autoimmune disease (Ramsey et al., 2002; Anderson et al., 2002). One of the few exceptions was the blindness most KO animals showed as a result of leukocyte and autoAb targeting of the retinal layer of rods and cones of the eye (Anderson et al., 2002). Thus, the strikingly aggressive development of type-1 diabetes in RIP-mOVA/OT-I double-tg mice carrying the Aire-null mutation in homozygous state serves as an important reinforcement of the concept that Aire is a critical factor in keeping autoimmune disease at bay. These mice were universally born with diabetes while their Aire wild-type counterparts showed no signs of hyperglycemia. Interestingly, RIP-mOVA/OT-II double-tg mice harboring homozygous Aire-null alleles showed no signs of diabetes. Several factors might predicate this difference: CD4<sup>+</sup>, rather than CD8<sup>+</sup>, T<sub>eff</sub> cells in play; escape of fewer T cells, or of cells displaying TCRs of lower affinity, into the periphery.

Clearly, then, Aire is an important factor in guarding against autoimmunity and, ultimately, autoimmune disease. Central mechanisms of tolerance induction are critical to the life of the individual and of the species. They do not, however, do their job alone. The autoimmune/inflammatory phenotypes reported for mice deficient in Foxp3 (Fontenot et al., 2003) are testament that peripheral tolerance induction mechanisms are also crucial.

### Experimental Procedures

#### Mice

Aire-deficient mice were derived and genotyped as previously described (Anderson et al., 2002). In the transfer and cotransfer experiments, donor mice were of a mixed Sv129/C57Bl/6 genetic background, derived from heterozygote x heterozygote crosses (F3xF3, F4xF4) of a backcross to the B6 background. Nude mice and RAG-1-deficient mice on a B6 background were used as recipients (The Jackson Laboratory, Bar Harbor, ME). HY TCR tg mice on the B6 background were obtained from Taconic (Germantown, MD). DEP TCR tg and hCRP tg mice on the B6 background (Klein et al., 1998) were kindly provided by Dr. Bruno Kyewski. OT-I and OT-II TCR tg mice on the B6 background were obtained from the Jackson Laboratory. RIP-mOVA tg mice in the B6 background (Kurts et al., 1996) were obtained from Dr. Andrew Lichtman, Harvard Medical School. All tg mice were genetically crossed to *Aire*<sup>+/-</sup> mice from an Sv129/B6 mixed background (backcross N3 or N4), with the exception of the OT-II animals, which were crossed to *Aire*<sup>+/-</sup> mice that had been backcrossed 5 generations to B6. HY TCR tg mice were genotyped by PCR as described (DeYoung et al., 2000). Mice were genotyped by using the primers listed in the supplement.

#### Real-Time PCR Analysis

Total RNA was prepared from whole spleen, whole thymus, or from cytofluorimetrically sorted thymic MECs from Aire knockout or wild-

type mice at 3–6 weeks of age. cDNA was made from total RNA, and real-time PCR using Taqman was carried out with the primers and probes listed in the supplement.

#### CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> Cell Populations

Splenocytes were harvested from 4- to 8-week-old Aire knockout or wild-type mice, and the red blood cell fraction eliminated by lysis. Splenocytes were stained on ice with allophycocyanin-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-CD25 and were then sorted on a MoFlo Flow Cytometer into CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cell populations. Sorted populations were reanalyzed and found to be greater than 90% pure. In vitro and in vivo suppression assays using these cells are described in the Supplemental Data.

#### Thymus Transplants

Thymi were isolated from newborn Aire knockout or wild-type mice, and were cultured in 1.35 mM 2-DG (Sigma, St. Louis, MO) for 6–8 days to deplete bone marrow-derived cells. The resulting thymic stromal cell preparation was washed in media for 2 hours, and single lobes from knockout or wild-type mice were transplanted or cotransplanted under the kidney capsule of adult (6–8 weeks of age) female nude mice of the B6 genotype. In the cotransplant experiments, knockout and wild-type thymus lobes were transplanted in the caudal and rostral ends of the kidney capsule. Animals were examined 10–12 weeks after thymus transplantation. Thymic tissue was found in the same orientation as at the time of transplant. In addition, stroma from individual harvested lobes was homogenized and RNA prepared. RT-PCR was performed to confirm the presence of the expected knockout or wild-type allele in the transplanted tissue, utilizing primers spanning the first to third exon of the *Aire* gene, as previously described (Anderson et al., 2002). Animals were taken for examination 10–12 weeks after transplantation. T cell reconstitution of transplanted mice was confirmed by total cell count and by cytofluorimetric analysis of the transplanted thymic lobes, staining for CD4 and CD8. Tissue from the salivary gland, stomach, liver, ovary, and eye were collected, fixed in 10% formalin, and embedded in paraffin. Sections were stained with H&E and were evaluated for the presence or absence of a mononuclear infiltrate in a blinded fashion.

#### Splenocyte Transfers

Red blood cell-depleted splenocytes were prepared from 8- to 10-week-old Aire knockout or wild-type mice. They were mixed as indicated, resuspended in phosphate-buffered saline, and injected into the tail vein of RAG-deficient B6 mice (The Jackson Laboratory) at the indicated cell numbers. Mice were harvested 10–12 weeks after transfer, and reconstitution with T cells and B cells was confirmed by staining with anti-CD4, anti-CD8, and B220 reagents. Tissue from the salivary gland, stomach, liver, ovary, and eye were collected, fixed in 10% formalin, and embedded in paraffin. Sections were stained with H&E and were evaluated for the presence or absence of a mononuclear infiltrate in a blinded fashion.

#### Antibodies and Flow Cytometry

The reagents used for cytofluorimetric analysis are listed in the supplementary materials. Stained cells were analyzed on an EPICS XL flow cytometer (Beckman Coulter) or MoFlo fluorescence activated cell sorter (Cytomation) and cell numbers were ascertained using a hemocytometer.

Analyses of HY, DEP<sup>xh</sup>CRP, and OT-II tg mice were performed at 5–8 weeks of age. (Only females with the DEP<sup>xh</sup>CRP tg mice were assessed because they make lower serum levels of hCRP, and consequently central deletion of DEP T cells is critically dependent on, though not necessarily directly mediated by, radioresistant thymic stromal cells [Klein et al., 1998].) OT-I transgenics were analyzed between birth and 2 days because of the incidence of diabetes in OT-I\*<sup>RIP</sup>-mOVA<sup>+</sup> Aire-deficient mice at birth and their subsequent death in the first few days of life.

Thymic MECs were sorted essentially according to (Anderson et al., 2002) for the phenotype CD45<sup>+</sup>EpCAM<sup>+</sup>Ly51<sup>int</sup>.

Cytofluorimetric analysis of intracellular Foxp3 levels was performed on B6/129 F2 wild-type and Aire-deficient mice of 3–5 weeks of age. Single-cell suspensions were prepared from thymus,

spleen and inguinal lymph node, and intracellular staining was performed according to the manufacturer's protocol (ebioscience).

#### Diabetes Assessment

OT-I\* and OT-I\*<sup>RIP</sup>-mOVA<sup>+</sup> transgenics were screened for diabetes by urine examination with Diastix test strips (Bayer Diagnostics). Whole blood of positive mice was tested for the presence of hyperglycemia on a blood glucometer (Glucometer Elite, Bayer Diagnostics). Diabetes was scored as positive in mice with blood glucose >250 mg/dl. In addition, whole pancreas was removed, fixed in 10% formalin, and embedded in paraffin. Tissue sections were stained with H&E.

#### Proliferation Assays

Proliferation assays using sorted MECs and OT-I responder cells are described in the Supplemental Data.

#### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two additional figures, and Supplemental References and can be found with this article online at <http://www.immunity.com/cgi/content/full/23/2/227/DC1/>.

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