European Journal of Immunology

Aire mediates thymic expression and tolerance of pancreatic antigens via an unconventional transcriptional mechanism

Dina Danso-Abeam[1,2], Kim A. Staats[3,4], Dean Franckaert[1,2], Ludo Van Den Bosch[3,4], Adrian Liston[1,2], Daniel H. D. Gray[5] and James Dooley*[1,2]*

[1] Autoimmune Genetics Laboratory, VIB, Leuven, Belgium
[2] Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium
[3] Vesalius Research Center, VIB and University of Leuven, Leuven, Belgium
[4] Laboratory for Neurobiology, University of Leuven, Leuven, Belgium
[5] Molecular Genetics of Cancer Division, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

The autoimmune regulator (Aire), mediates central tolerance of peripheral self. Its activity in thymic epithelial cells (TECs) directs the ectopic expression of thousands of tissue-restricted antigens (TRAs), causing the deletion of autoreactive thymocytes. The molecular mechanisms orchestrating the breadth of transcriptional regulation by Aire remain unknown. One prominent model capable of explaining both the uniquely high number of Aire-dependent targets and their specificity posits that tissue-specific transcription factors induced by Aire directly activate their canonical targets, exponentially adding to the total number of Aire-dependent TRAs. To test this "Hierarchical Transcription" model, we analysed mice deficient in the pancreatic master transcription factor pancreatic and duodenal homeobox 1 (Pdx1), specifically in TECs ($Pdx1^{\Delta Foxn1}$), for the expression and tolerance of pancreatic TRAs. Surprisingly, we found that lack of Pdx1 in TECs did not reduce the transcription of insulin or somatostatin, or alter glucagon expression. Moreover, in a model of thymic deletion driven by a neo-TRA under the control of the insulin promoter, Pdx1 in TECs was not required to affect thymocyte deletion or the generation of regulatory T (Treg) cells. These findings suggest that the capacity of Aire to regulate expression of a huge array of TRAs relies solely on an unconventional transcriptional mechanism, without intermediary transcription factors.

Keywords: Aire · Immune tolerance · Negative selection · Thymic epithelium

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

The autoimmune regulator (Aire) induces the expression of thousands of tissue restricted antigens (TRAs) in thymic epithelial cells (TECs) [1]. By doing so, Aire promotes clonal deletion of differentiating T cells that recognize TRAs, thereby preventing autoimmune disease [2]. Deficiency in *Aire* results in an autoimmune condition called Autoimmune Polyendocrinopathy

Correspondence: Dr. James Dooley
e-mail: James.Dooley@vib-kuleuven.be

*These authors contributed equally to this work.

Syndrome type 1, with a diagnostic clinical triad of candidiasis, adrenal insufficiency and hypoparathyroidism [3]. These symptoms are accompanied by secondary autoimmune manifestations, such as Type I Diabetes (T1D) or gastritis, and most patients develop autoantibodies against TRAs expressed in the affected tissues (reviewed in [4] and [5]). Although this condition is relatively rare, defects in the same pathway also contribute to the development of more common autoimmune diseases, such as the progression to autoimmune myasthenia gravis [6] and the risk of T1D caused by polymorphisms that reduce thymic expression of insulin [7].

Despite the importance of Aire to immunological tolerance, precisely how Aire mediates the ectopic transcription of thousands of TRAs remains unresolved. Various models of transcriptional regulation have been proposed, ranging from (i) direct DNA binding [8], (ii) non-specific activation of genes within genomic regions [9], (iii) gain of promoter specificity via interaction partners [5] or (iv) recognition of a histone tag left by other proteins ([10], reviewed in [11]). None of these models can fully explain how Aire affects expression of potentially thousands of genes with vastly different modes of regulation in the respective autochthonic tissues, and yet maintains cell-type specificity in the range of genes targeted [12, 13]. Furthermore, chromosomal context models are not consistent with the observation that transgenes driven from promoters of known Aire targets (such as insulin) remain Aire-dependent when inserted in alternative chromosomal positions [2, 14, 15]. Therefore, attention has turned to hybrid models, where Aire recruits additional proteins to execute complementary functions. Abramson et al. recently defined many of the molecular partners of Aire involved in its function in gene regulation [16] and the ability of Aire to preferentially activate genes via stalled RNA polymerase at the transcription start site has been elucidated by Giraud et al. [17]. However, a significant subset of Aire targets may be induced indirectly [9]. It has been shown that Aire activates the expression of several transcription factors [18]. These, in turn, might mediate the expression of many apparently "Aire-dependent" TRAs, greatly expanding the total number of Aire-dependent targets by adding secondary targets to the direct primary targets. Here, we investigate whether such a mechanism regulates transcription and tolerance of pancreas-specific antigens in the thymus.

Expression of insulin by medullary TECs (mTECs) is necessary to prevent T1D [19] and polymorphisms in the insulin gene that reduce its expression by mTECs represent a significant risk factor for T1D [7]. Thymic expression of insulin is absolutely Aire dependent [18]; however in the pancreas, Pdx1 (pancreatic and duodenal homeobox 1) drives the expression of islet-specific genes such as insulin [20] and somatostatin (Sst) [21–23]. Interestingly, Pdx1 is also expressed by mTECs in an Aire-dependent manner, raising the possibility that insulin expression in the thymus is downstream of Aire-dependent Pdx1 expression, and thus a secondary target [23]. Therefore, the expression of insulin by mTECs represents an ideal prototypical TRA to probe the "Hierarchical Transcription" model. In this study, we generated mice that specifically lack Pdx1 in TECs only (referred to as $Pdx1^{\Delta Foxn1}$) to investigate whether Aire-mediated Pdx1 expression is necessary for insulin transcription. We observed that, although insulin expression was absent in mTECs from $Aire^{-/-}$ mice, insulin expression persisted in the mTECs of the $Pdx1^{\Delta Foxn1}$ mice. Furthermore, we found that transcription mediated by the insulin promoter could still impose immunological tolerance, despite the lack of Pdx1 in mTECs. These observations indicate that Aire-dependent TRA expression for thymic tolerance is independent of the molecular pathways utilized during autochthonic expression, and suggest that Aire requires primary recognition of the regulatory regions to a wide variety of target genes.

Results

A new mouse strain with thymus-specific Pdx1-deficiency and normal Aire expression

To investigate the requirement of Pdx1 for thymic insulin expression, we devised a mouse model system whereby Pdx1 expression was specifically ablated in TECs. Intercrosses of $Foxn1^{Cre}$ [24] and floxed *Pdx1* [25] mice generated $Foxn1^{Cre}$ $Pdx1^{fl/fl}$ mice (henceforth termed $Pdx1^{\Delta Foxn1}$) that have *Pdx1* deleted from all TECs, while retaining normal expression in the pancreas to prevent diabetes induction [26] and consequent thymic atrophy. An important control was provided by $Aire^{-/-}$ mice [18], since Pdx1 expression is Aire dependent [23]. In order to interpret the requirement of Pdx1 for insulin expression by TECs, it was important to establish whether the converse was true; i.e. was Aire expression changed by Pdx-1 deficiency? We sort purified cortical and mTECs from the two strains and their WT siblings. We observed that Aire expression was restricted to mTECs, was absent in mTECs from the $Aire^{-/-}$ mice, but remained at normal levels in the $Pdx1^{\Delta Foxn1}$ mice as detected by quantitative PCR (qPCR; Fig. 1A). Importantly, we detected normal protein levels and distribution of Aire in the thymi of $Pdx1^{\Delta Foxn1}$ mice by immunohistology (Fig. 1C), firmly establishing that Aire expression is unaffected by Pdx1-deficiency in the thymus. We next assayed the relative expression of Pdx1 and found that, while mTECs from WT mice showed significant levels of *Pdx1* transcription, no transcript was detected in mTECS from $Aire^{-/-}$ and $Pdx1^{\Delta Foxn1}$ mice (Fig. 1B). These observations confirmed that thymic Pdx1 expression (i) was restricted to mTECs, (ii) was Aire-dependent, (iii) was completely absent from TECs in $Pdx1^{\Delta Foxn1}$, and (iv) was not required for Aire expression. With this validation of our experimental system, we went on to assay the competence of $Pdx1^{\Delta Foxn1}$ mice for the thymic expression of pancreas-specific genes.

Pdx-1 expression by TECs is not necessary for insulin transcription

We next sought to test the hypothesis that Aire utilized the direct target Pdx1 as an intermediate in the expression program for pancreatic TRAs. We assayed four pancreatic genes that are

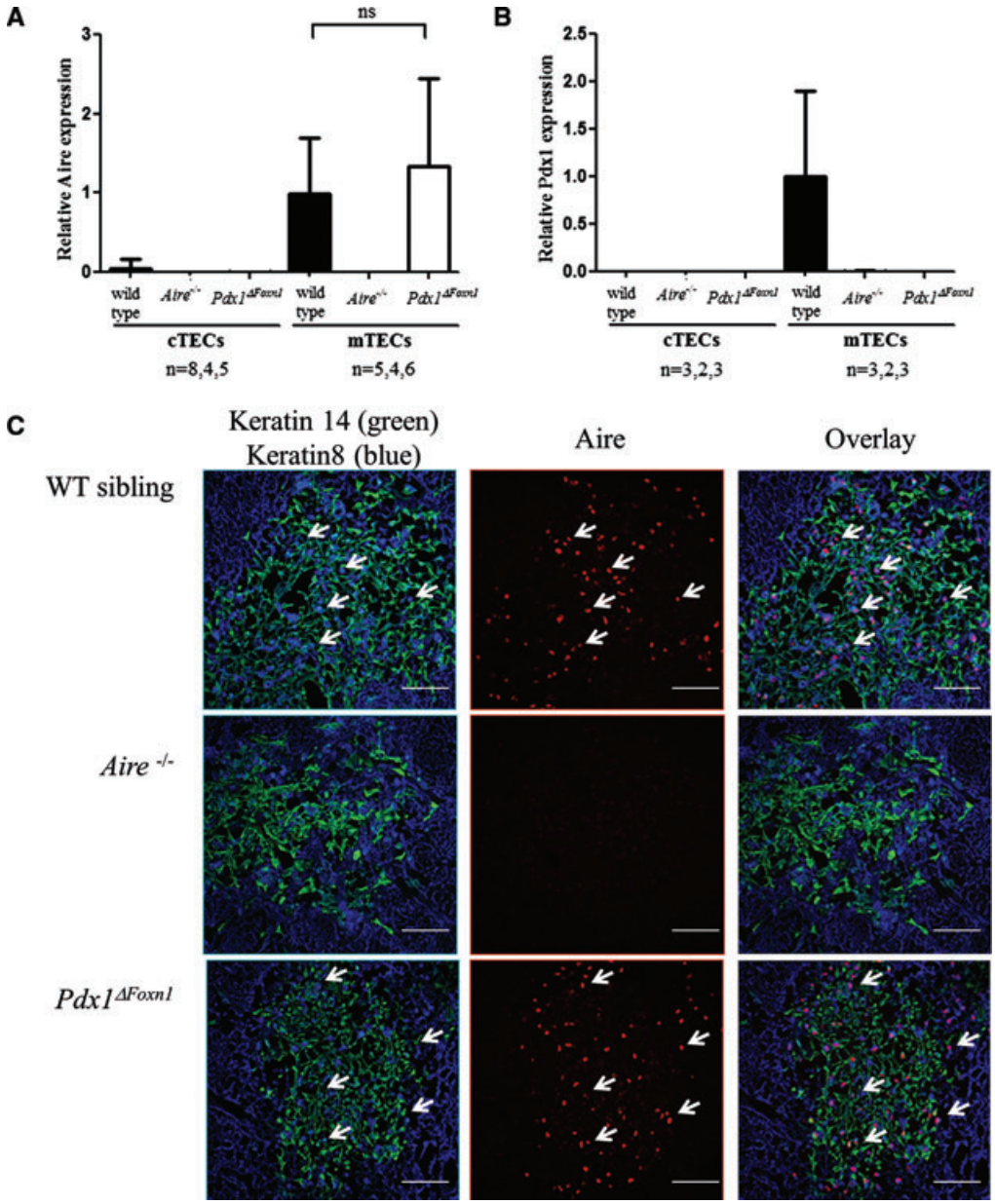

Figure 1. Expression of Aire and Pdx1 mRNA in TECs from WT, *Aire*$^{-/-}$ and *Pdx1*$^{\Delta Foxn1}$ mice. TEC subsets were purified from WT (black bars), *Aire*$^{-/-}$ (not detected) or *Pdx1*$^{\Delta Foxn1}$ (white bars) mice and (A) Aire and (B) Pdx1 transcripts were assayed by qPCR. Data are shown as the gene expression relative to WT levels and are presented as the mean + SD of the indicated number of biological replicates, each performed in two technical replicates. Data shown are pooled from three experiments performed. (C) Immunofluorescent staining of thymus sections from WT, *Aire*$^{-/-}$ and *Pdx1*$^{\Delta Foxn1}$ mice, representative of three experiments. Keratin 14 (green) and keratin 8 (blue) are shown in left panels, Aire (red) in centre and an overlay in the right panels. Arrows indicate examples of mTECs expressing Aire. Scale bars, 100 μm. Autoimmune regulator (*Aire*), Pancreatic and duodenal homeobox 1 (*Pdx1*).

differentially regulated by Pdx1 and are expressed by TECs: *Ins2* and *Sst* are induced by Pdx1, *Gcg* (glucagon) repressed, while *Gad1* (glutamate decarboxylase 1) is unaffected [27]. We found that *Ins2* (Fig. 2A) and *Sst* (Fig. 2B) transcripts were detected at normal levels in mTECs from WT and *Pdx1*$^{\Delta Foxn1}$ mice, but were absent in mTECs from *Aire*$^{-/-}$ mice. Likewise, transcription of the Aire-dependent pancreatic TRA, *Gcg*, was not altered by the loss of Pdx-1 from TECs (Fig. 2C). Importantly, we could detect *Gcg* protein expressed by TECs from *Pdx1*$^{\Delta Foxn1}$ mice at similar levels and distribution as WT (Fig. 2E), supporting the transcriptional analysis. Interestingly, the expression of Aire and *Gcg* generally did not overlap (Fig. 2E), suggesting that, although *Gcg* is an Aire-dependent TRA, its expression is temporally distinct from Aire detected in this manner. In addition, we found that Gad1 expression was not influenced by either Aire deficiency (consistent with [27]) or Pdx1 deficiency (Fig. 2D). Collectively, these results indicate that Pdx1 in TECs does not affect the expression of islet TRAs normally regulated by Pdx1 in the pancreas.

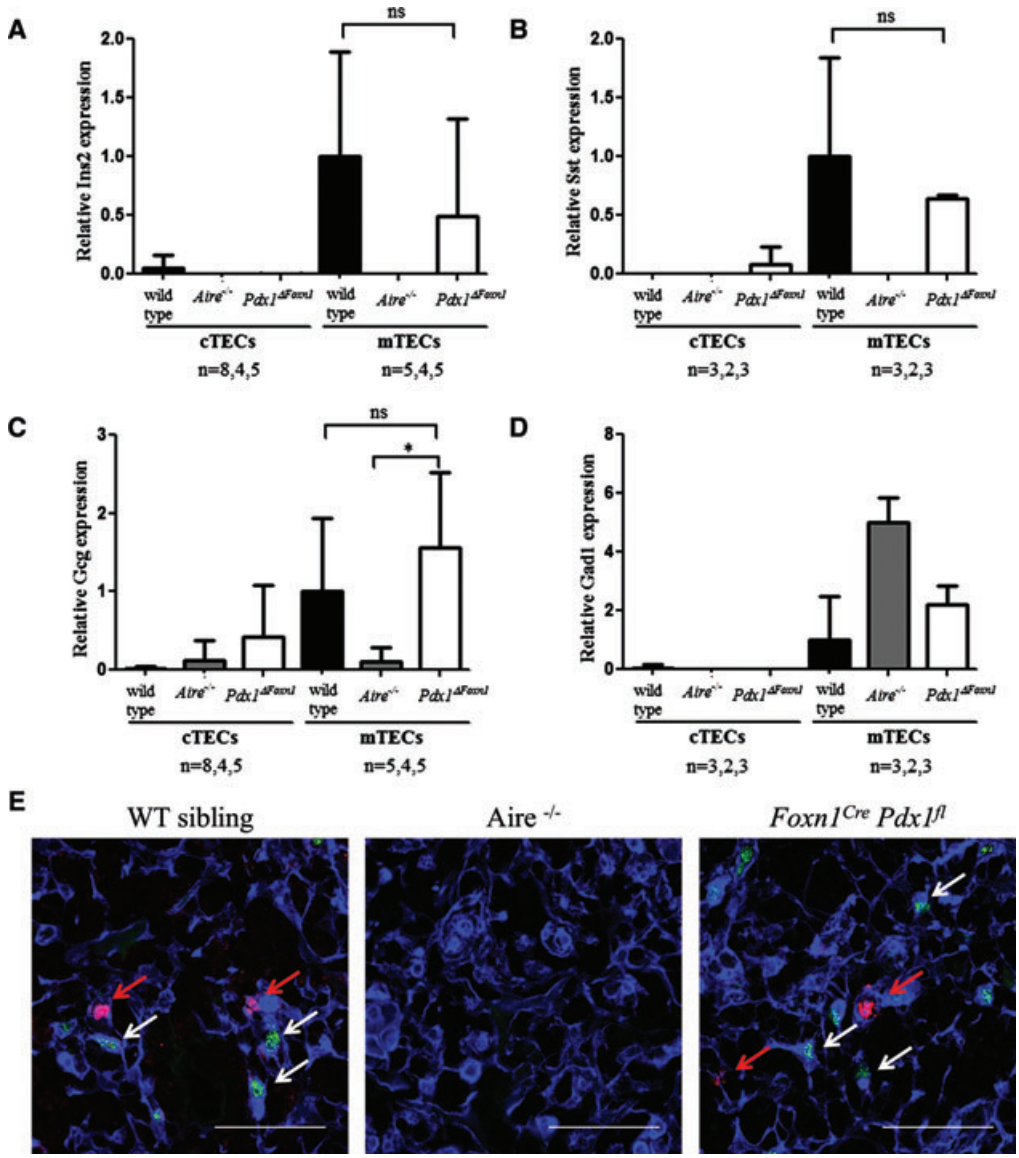

Figure 2. Expression of pancreatic TRAs is Pdx1-independent in mTECs. **(A–C)** qPCR analysis of Aire-dependent, islet-specific TRA (A) *Ins2*, (B) *Sst* and (C) *Gcg* transcripts and (D) an Aire-independent TRA (*Gad1*) in cTECs and mTECs purified from WT (black bars), $Aire^{-/-}$ (grey bars) and $Pdx1^{\Delta Foxn1}$ mice (white bars). Data are shown as the gene expression relative to the WT mTEC levels and are presented as the mean + SD of the indicated number of biological replicates, each performed in two technical replicates. Data shown are pooled from three experiments performed. $^{*}p < 0.05$, Student's *t* test. (E) Immunofluorescence staining of thymi from WT, $Aire^{-/-}$ and $Pdx1^{\Delta Foxn1}$ mice, representative of three experiments. Red arrows indicate Gcg^{+} cells and white arrows indicate $Aire^{+}$ cells. Blue, Keratin 8; green, Aire; red,*Gcg*. Scale bars, 25 μm. Autoimmune regulator (*Aire*), Pancreatic and duodenal homeobox 1 (*Pdx1*), insulin (*Ins2*), *Sst* and *Gcg*, glutamic acid decarboxylase 1 (*Gad1*).

Thymic expression of Pdx1 is not required for T-cell tolerance against pancreatic TRA

The negative selection of autoreactive thymocytes is very sensitive to minor changes in Aire-dependent TRAs [28]. To extend our expression data to T-cell tolerance, we used a sensitive model of negative selection driven by the insulin promoter. The insulin promoter-driven hen egg lysozyme (insHEL) transgenic mouse expresses the model antigen, hen egg lysozyme (HEL), in mTECs under the influence of the rat insulin promoter in an Aire-dependent manner [28]. In the 3A9 TCR transgenic model, T cells express a TCR reactive to a fragment of HEL presented by MHC class II H2-A^{k} molecules [29]. Taken together, the insHEL and 3A9 TCR transgenics create a negative selection model, where the Aire-dependent expression of insHEL by mTECs causes the apoptotic deletion of CD4SP thymocytes expressing the 3A9 TCR [28]. We bred $Pdx1^{\Delta Foxn1}$ insHEL transgenic mice and sorted mTECs from $Pdx1^{wt}$ insHEL and $Pdx1^{\Delta Foxn1}$insHEL mice. Expression of insHEL was not reduced in mTECs from $Pdx1^{\Delta Foxn1}$ insHEL mice compared with those from $Pdx1^{wt}$ insHEL mice (Supporting Information Fig. 1), unlike what was previously observed with $Aire^{-/-}$ insHEL mice [28]. However, as the value of the insHEL 3A9 system

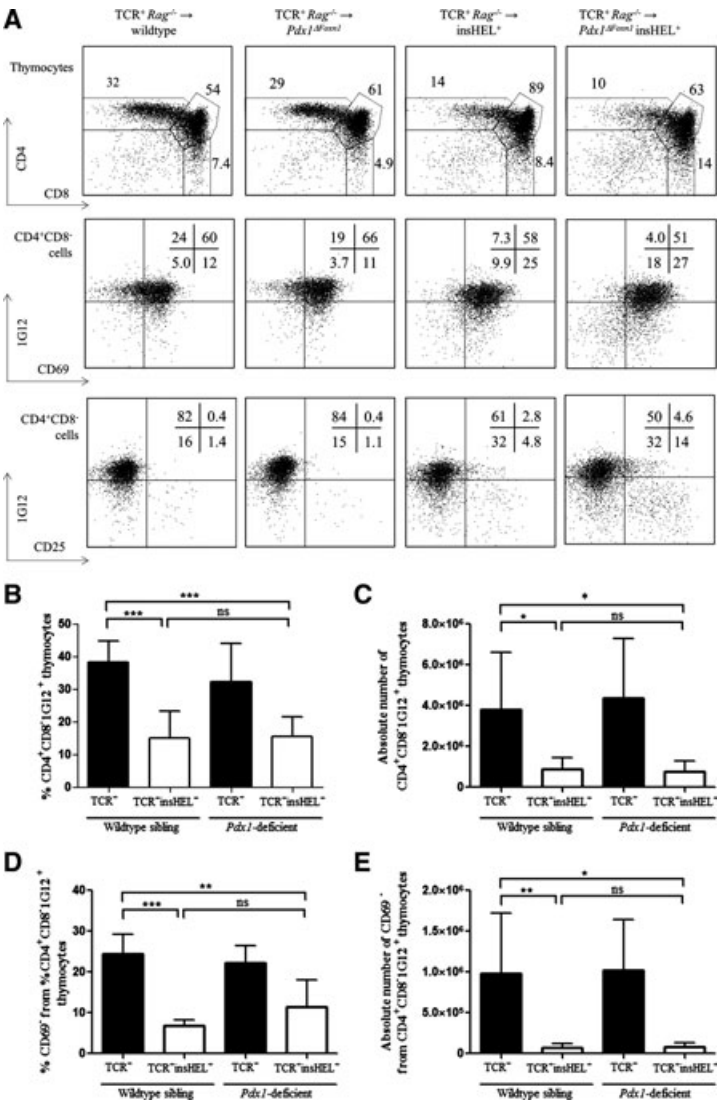

Figure 3. Deletion of insHEL-reactive thymocytes is independent of thymic *Pdx1*. Mice of the indicated genotypes were reconstituted with *Rag1*$^{-/-}$ 3A9 TCR transgenic bone marrow and analysed 8 weeks later. (A) Representative dot plots of CD4 versus CD8 (top), 1G12 versus CD69 (middle) and 1G12 versus CD25 (bottom) expression gated on the indicated thymocyte populations. The numbers shown are the proportions of gated cells. (B–E) The mean (B and D) percentage and (C and E) number of (B and C) CD4SP1G12^{+} and (D and E) mature CD69^{-}CD4SP1G12^{+} thymocytes of age-matched mice of the indicated genotypes are shown. Data are shown as the mean number of cells from insHEL-negative mice (black bars) and insHEL-positive transgenic (white bars) mice. Error bars indicate standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Student's *t*-test. Data shown are representative of two independent experiments.

is in measuring defects in the physiologically more valid outcome of thymic negative selection, we bred the *Pdx1*$^{\Delta Foxn1}$ insHEL mice on the *Rag1*$^{-/-}$.B10.Br background and, along with controls, created haematopoietic chimeras using bone marrow from 3A9 TCR transgenic *Rag1*$^{-/-}$ mice (*Rag1*-deficiency prevents endogenous rearrangement of TCR alpha chains). The fate of HEL-specific (autoreactive) thymocytes was then tracked by flow cytometry using the 3A9 TCR clonotype-specific 1G12 antibody [30]. In insHEL-negative recipients of both genotypes, we observed comparable numbers of positively selected HEL-specific CD4^{+}CD8^{-} (CD4 single positive, CD4SP) thymocytes, indicating that the loss of Pdx1 had no effect on positive selection (Fig. 3A and B). The positively selected CD4SP thymocytes from *Pdx1*$^{\Delta Foxn1}$ mice exhibited normal maturation, with similar downregulation of CD69 to WT chimeras (Fig. 3A, D and Table 1).

After confirming that the loss of Pdx1 in the thymus had no influence on positive selection, we next investigated whether Pdx1 played a role in central tolerance against insHEL. As previously demonstrated [2], WT insHEL-positive chimeras had a large decrease in the proportion and numbers of 3A9 CD4SP thymocytes compared with those in the WT insHEL-negative chimeras (Fig. 3B, C), representing the impact of deletion against this model TRA [2]. This effect was even more pronounced in the CD69^{-} mature fraction of 3A9 TCR CD4SP thymocytes, which are preferentially deleted in this model [2] (Fig. 3D).

Under negative selecting conditions, we found no difference in the number or proportion of 3A9 TCR CD4SP cells in the thymus of the *Pdx1*$^{\Delta Foxn1}$ insHEL-positive chimeras compared to WT insHEL-positive chimeras (Fig. 3A and B). Furthermore, normal deletion of 3A9 TCR CD4SP thymocytes was observed in *Pdx1*$^{\Delta Foxn1}$ insHEL chimeras, as the proportion (Fig. 3D) and number (Fig. 3E) of CD69^{-} 1G12^{+} CD4SP was equivalent to WT insHEL chimeras (Table 1). These data indicate that efficient negative selection induced by insHEL was not modified by the loss of Pdx1 in mTECs. Therefore, although Pdx1 was absent from mTECs (Fig. 1B), the insulin promoter remained active, driving the expression of HEL in the *Pdx1*$^{\Delta Foxn1}$ thymus and deletion of 3A9 TCR thymocytes.

Table 1. Effect of thymic Pdx1-deficiency on positive selection and negative selection of pancreatic-specific autoreactive T cells

Bone marrow donor	TCR$^+$ *Rag*$^{-/-}$	TCR$^+$ *Rag*$^{-/-}$	TCR$^+$ *Rag*$^{-/-}$	TCR$^+$ *Rag*$^{-/-}$
Bone marrow recipient	Wildtype	insHEL$^+$	*Pdx1*$^{\Delta Foxn1}$	*Pdx1*$^{\Delta Foxn1}$ insHEL$^+$
Thymus				
n	6	7	5	5
Percentage of DN	7.2 ± 1.4[a)]	14 ± 3.5***	7.1 ± 2.0	15 ± 1.7***
Percentage of DP	41 ± 10	49 ± 16	45 ± 8.9	40 ± 7.1
Percentage of CD4 SP	40 ± 6.4	18 ± 7.7***	34 ± 11	118 ± 6.4 ***
Percentage of IG12$^+$	96 ± 1.0	78 ± 21	93 ± 7.4	89 ± 7.2[b)]
Percentage of IG12$^+$CD69$^-$	25 ± 4.7	6.9 ± 1.4 ***	22 ± 4.2	12 ± 6.6[c)]
Percentage of CD25$^+$	1.9 ± 0.5	12.8 ± 7.9[c)]	2.4± 1.2	14.7 ± 4.8 ***
Percentage of CD8 SP	9.7 ± 2.3	15 ± 6.3	10.8 ±2.5	21.1 ± 3.8***
Spleen				
n	7	7	5	5
Percentage of B220$^+$	5.1 ± 4.3	6.0 ± 3.1	4.0 ± 1.9	8.3 ± 7.2
Percentage of CD4$^+$	40 ± 7.6	26 ± 8.4[c)]	42 ± 7.0	30 ± 8.0[b)]
Percentage of IG12$^+$	83 ± 15	43 ± 29[c)]	69 ± 18	64 ± 26
Percentage of CD4$^+$IG12$^+$	40 ± 13	13 ± 11[c)]	30 ± 9.7	21 ± 12[b)]
Percentage of CD62L$^-$	17 ± 5.4	36 ± 13[c)]	15 ± 5.4	37 ± 11[c)]
Percentage of CD8$^+$	18 ± 5.3	16 ± 4.3	22 ± 3.9	16 ± 3.9

[a)] Mean ± SD.
[b)] $p < 0.05$ versus TCR$^+$ *Rag*$^{-/-}$ into WT.
[c)] $p < 0.01$ versus TCR$^+$ *Rag*$^{-/-}$ into WT.
*** $p < 0.001$ versus TCR$^+$ *Rag*$^{-/-}$ into WT.

Although Pdx1 deficiency affected neither positive nor negative selection of thymocytes, it was important to determine whether it might influence the regulatory T (Treg)-cell compartment. To assay Treg-cell development, we analysed the expression of CD25 among the CD4SP T cells in the bone-marrow chimeras outlined above. It is currently held that CD25$^+$ Treg cells arise from self-reactive thymocytes that avoid deletion and have been reported to have a higher representation in HEL$^+$ mice with Aire-competent mTECs [31,32]. Consistent with this report, we observed a higher percentage turnout of CD25$^+$ thymocytes from the HEL$^+$ mice compared with that of their siblings without the HEL transgene (Supporting Information Fig. 2A and Table 1). However, no significant difference was observed between any of the groups when absolute cell count was considered (Supporting Information Fig. 2B). Additionally, we found that *Pdx1*$^{\Delta Foxn1}$insHEL$^+$ mice had an average of 14.7% (±4.8%) CD25$^+$ HEL-reactive T cells, similar to the mean percentage of 12.8% (±7.9%) observed in WT insHEL$^+$ mice (Supporting Information Fig. 2A and Table 1). These data suggest that the loss of thymic Pdx1 did not influence thymic Treg-cell production.

Finally, we determined whether Pdx1 deficiency in TECs influenced the peripheral outcome of autoreactive circulating T cells that escape thymic deletion. We observed significantly a lower percentages of circulating CD4$^+$1G12$^+$ cells in the spleens of the 3A9 TCR/insHEL double transgenics compared with that in their insHEL-negative counterparts (due to thymic negative selection; Fig. 4A and B). Double transgenic *Pdx1*$^{\Delta Foxn1}$ mice showed similar proportions and numbers of splenic CD4$^+$1G12$^+$ T cells as WT 3A9 TCR/insHEL controls (Fig. 4A and B). Of those CD4$^+$1G12$^+$ T cells that emerged in the periphery of 3A9 TCR/insHEL (regardless of Pdx1 status), a higher percentage had downregulated CD62L (a measure of antigen experience), compared to insHEL-negative chimeras (Fig. 4C and Table 1). In summary, consistent with the insulin expression data, TEC-specific ablation of Pdx1 did not impair insHEL-mediated central tolerance.

Discussion

The mechanism by which Aire mediates the transcription of thousands of TRAs in mTECs is an important question in the field of autoimmunity [16, 33]. Direct transcriptional regulation by Aire seems to be via an unconventional mechanism, however a "Hierarchical" model might explain the breadth of genes affected when Aire-dependent TRAs are envisaged as transcriptional activators capable of driving the expression of other downstream TRAs [11]. We tested a version of this model that postulates that conserved transcription factors play an intermediate role in the regulation of pancreatic TRAs. The Aire-dependent transcription of insulin in mTECs is required for central tolerance and prevention of T1D [19]. Interestingly, the pancreatic transcription factor, Pdx1, which is necessary and sufficient for insulin expression in pancreatic islets, is also dependent on Aire for its expression in the thymus [23]. We therefore hypothesized that Aire might indirectly regulate islet-specific genes in the thymus by inducing the expression of Pdx1, which then directly activates the expression of downstream TRAs, such as insulin and Sst. We tested this hypothesis using a mouse model that deleted *Pdx1* only from the thymus and

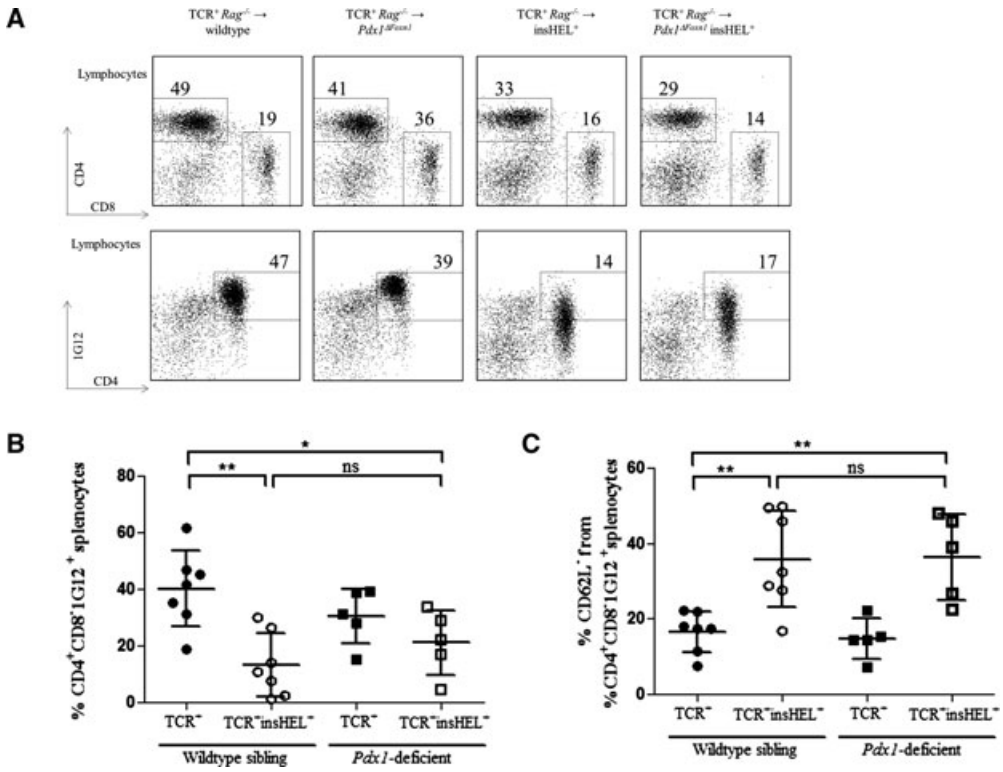

Figure 4. Pdx1-deficient mice exhibit equivalent negative selection to Pdx1-sufficient mice and it is mirrored in the periphery. Splenocytes from mice reconstituted with TCR transgenic bone marrow were stained with 1G12 anti-TCR clonotypic antibody and differentiation/activation marker. (A) Representative profiles of the indicated genotypes are shown. The numbers shown indicate the percentages of gated cells that fall within the indicated regions. (B) CD4 SP splenocytes and (C) the proportion that exhibited activated phenotype, i.e. $1G12^{+}CD62L^{-}$. Each symbol represents an individual mouse and bars represent the mean values from pooled age-matched siblings of the indicated genotypes. Data are shown as the mean number of cells from insHEL WT mice (black symbols) and from insHEL transgenic siblings (white symbols). Error bars indicate standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Student's *t*-test. Data shown are representative of two independent experiments.

investigated TRA expression and the induction of central tolerance to insulin.

Our qPCR analysis of *Ins2* and *Sst* transcription (both induced by Pdx1 in the pancreas) indicated that Pdx1 in mTECs was dispensable as an intermediate transcription factor. Similarly, our results indicated that loss of Pdx1 (which normally inhibits beta cell expression of *Gcg* [34]) did not influence *Gcg* transcription in mTECs. These data indicate that *Ins2*, *Sst* and *Gcg* regulation in the thymus is independent of Pdx1. These results build upon a previous report from Villasenor et al. [33] that analysed *Ins2* transcription in extracts of whole thymus from embryonic or perinatal $Pdx1^{-/-}$ mice. Under these conditions, they found no difference in thymic *Ins2* transcription in $Pdx1^{-/-}$ or $cMaf^{-/-}$ mice, although it is notable that this time-point is prior to maximal Aire expression and TRA measurement in whole thymus is not considered robust without the use of an Aire-deficient control. Here, we extend upon this study to show that transcription of *Ins2*, *Sst*, *Gcg* and *Gad1* measured in purified TEC subsets was unaltered by Pdx1 deficiency specifically in the TEC compartment of a mature thymic microenvironment. In addition, we found that normal Aire expression was also maintained in $Pdx1^{\Delta Foxn1}$ mTEC. Importantly, we also showed that the protein levels and distribution of a pancreatic TRA (*Gcg*) did not change in the absence of Pdx1 and the physiological outcome of immunological tolerance against a neo-TRA (insHEL) was not reduced.

An interesting observation from our immunohistological analysis of *Gcg* expression was that this Aire-dependent TRA did not co-localize with Aire-positive mTECs. This finding suggests that TRA expression may be temporally or spatially separate of Aire's activity as a transcriptional regulator. Although we cannot rule out that a low level of Aire protein that is undetectable via this approach may indeed be present in *Gcg*-expressing mTECs, Villasenor made a similar finding by single-cell transcriptional analysis of *Ins2* and *Aire* [33]. Therefore, one interpretation of these data is that Aire expression in mTECs is transient, but somehow induces enduring transcriptional activation of TRAs after it has been downregulated. This notion is at odds with current models that posit that Aire is expressed in terminally differentiated mTECs [9, 35].

The expression of TRAs in the thymus promotes the deletion of autoreactive thymocytes to preserve tolerance. For example, the dysregulation of insulin expression in TECs predisposes mice and humans to autoimmune disorders such as T1D [7, 19]. In a further test of the requirement for Pdx1 in Aire-dependent tolerance, we examined the efficiency of thymocyte deletion driven by an insulin promoter in $Pdx1^{\Delta Foxn1}$ mice. Our results show that efficient deletion of autoreactive insHEL-specific thymocytes does not require

Pdx1 expression by mTECs. This finding is consistent with our TRA expression data and also shows that Pdx1 has no impact on the antigen presentation of pancreatic TRA.

Collectively, our data indicate that other, Pdx1-independent, mechanisms drive pancreatic TRA expression in the thymus. Therefore, Pdx1 expression by TECs likely represents another TRA to purge the thymus of Pdx1-reactive thymocytes, rather than the expression of a functional transcription factor. Two possible reasons for the inability of Pdx1 to affect transcriptional changes are that (i) the Pdx1 protein might be processed for antigen presentation too quickly; and/or (ii) the protein does not translocate to the nucleus to serve as a transcription factor.

Altogether, our data support the notion that Aire is directly required for the thymic expression of TRAs. Although Aire drives the transcription of Pdx1 in the thymus, this expression is not necessary for tolerogenic insulin expression in the thymus. This finding suggests that Aire does not use the conserved Pdx1-dependent peripheral regulation pathways in the thymus, and supports a model for direct transcriptional activity of Aire on Aire-regulated genes in the thymus.

Materials and methods

Mice

All mice were kept in the animal facility of the KU Leuven and all experiments were in accordance with ethical protocols approved by KU Leuven. Experimental mice were age-matched and their genotypes were confirmed by PCR before being included in the study. The Aire deficient (*Aire*$^{-/-}$) [18], 3A9 TCR transgenic [29], insHEL transgenic [36], *Foxn1*Cre [24], *Pdx1*$^{fl/fl}$ [25] and *Rag1* knockout *(Rag1*$^{-/-}$*)* [37] mice used in this project have been previously described. The 3A9 TCR and insHEL mice were backcrossed eight times to the C57Bl/10.Br genetic background while the rest of the mice were backcrossed more than 8 generations to the C57Bl/6 background. *Pdx1*$^{\Delta Foxn1}$ *Rag*$^{-/-}$ insHEL mice and *Pdx1*^{wt} *Rag*$^{-/-}$ insHEL siblings were used on a mixed C57Bl/10xC57Bl/6 background fixed for H2-A^{k}.

Adult mice (8 to 11 weeks old) were irradiated with a single dose of 9 Gy using a LiNac system (linear accelerator, 6MV photons, Varian Medical Systems, Palo Alto, CA). Mice were reconstituted with 1×10^6 bone marrow cells depleted of T cells using CD4 (GK1.5) and CD8 (2.43), both are kind gifts from A. Farr, University of Washington, and Low-Tox M Rabbit Complement (Cedarlane). Recipients were maintained on Baytril for 4 weeks, their blood glucose was monitored 6 weeks after reconstitution and subsequent analysis at 8 weeks.

Flow cytometric analysis and immunohistology

Thymocytes and splenocytes were stained with combinations of the following antibodies (all from eBiosciences, unless otherwise noted): CD8 PE-Cy5, CD62L PE, CD69 PE-Cy7, B220 PE-Cy5, CD25 FITC and CD4 allophycocyanin-Cy7 (BD Biosciences). Anti-IgG1 allophycocyanin (BD Biosciences) was used as a secondary stain to detect 1G12 supernatant reactivity. Stained cell suspensions were analysed on a CANTO I (Becton Dickinson) instrument. For immunohistology, sections were prepared from thymi and stained as previously outlined [38]. Staining was performed using the following antibodies: anti-mouse keratin 8 (Troma-1; Developmental Studies Hybridoma Bank), anti-mouse keratin 14 (PRB-115P-100; Covance), Rabbit anti-Aire (M-300; Santa Cruz) and Goat anti-*Gcg* (N-17; Santa Cruz). The following detection antibodies were used: Donkey anti-Rabbit 488, Donkey anti-Goat 546, and Chicken anti-Rat 647 (Molecular probes). Images were acquired with an LSM 510 Meta confocal microscope (Zeiss).

TEC purification

Single cell suspensions of thymic stromal cells were isolated following previously described protocols [39, 40], with few modifications. Briefly, thymi from five to six mice were trimmed of fat and other connective tissue. Thymic lobes were minced in RPMI-1640 medium (Invitrogen) with tweezers and scalpel blades. Thymocytes were flushed with RPMI-1640 and thymic fragments were then digested with collagenase D (Roche Applied Science), followed by Dispase/collagenase (Dispase from Roche Applied Science) for three to four times or until all fragments were completely digested. The collagenase digestion was carried out at room temperature with gentle agitation and the Dispase-collagenase digestion performed at 34°C, also with gentle agitation. After filtration through a 100 μm filter, the TEC-enriched fractions were washed with FACS buffer (20 mM EDTA PBS + 2% FBS + 0.1% NaN_3), counted and stained with the following conjugates: FITC–conjugated Ulex Europaeus Agglutinin I (Vector Labs), PE-conjugated anti-BP-1 (6C3; eBioscience), PerCP Cy 5.5–conjugated anti–H-2A/H-2E (M5/114; Biolegend), allophycocyanin-conjugated anti-CD45 (104; eBiosciences) and PE-Cy7-conjugated anti-Epcam (G8.8; Biolegend). Cells were sorted into TRIzol (Invitrogen) using a FACSAria instrument (Becton Dickinson). Cells sorted were defined by the following phenotypes: cortical TECs (cTECs): CD45$^-$MHCII$^+$G8.8$^+$BP-1$^+$UEA$^-$ and mTECs: CD45$^-$MHCII$^+$G8.8$^+$BP-1$^-$UEA$^+$ (representative FACS plot in Supporting Information Fig. 3). Sorted cells were stored at –80°C until used for mRNA extraction.

Real time qPCR

mTECs and cTECs were sorted into TRIzol and total RNA isolated using RNeasy micro kit (Qiagen) and reverse transcribed with random hexamers (Life Technologies) and M-MLV (Invitrogen). qPCR was performed with the StepOnePlus (Life Technologies) and TaqMan Fast Universal PCR Master Mix (Life Technologies). The following assays were used: Aire (Mm.PT.47.5899927, IDT DNA), Pdx1 (Mm00435565_m1, Life

Technologies), Insulin2 (Mm.47.17321456, IDT DNA), Sst (Mm.PT.49a.7678291, IDT DNA), *Gcg* (Mm.PT.47.17201536, IDT DNA), Gad1 (Mm.PT.49a.11296402, IDT DNA) and GAPDH (Mm99999915_g1, Life Technologies). HEL was measured by SYBR Select Master Mix (Life Technologies) using the primers 5′ TCGGTACCCTTGCAGCGGTT and GAGCGTGAACTGCGCGAAGA. Relative gene expression was determined by the $2^{-\Delta\Delta ct}$ method [41] and normalized to the average of the WT mTECs group.

Statistical analysis

Student's *t*-test was used to compare differences between means with GraphPad Prism 5 software. All error bars were defined by standard deviation. The difference between two groups was considered significant if the *p*-value was less than 0.05.

Acknowledgements: We would like to gratefully acknowledge Dr. A. Farr for the provision of hybridoma clones. This work was funded by grants from the VIB and FWO and an NH&MRC Project Grant (no. 637332). D.H.D.G was supported by an NH&MRC Career Development Fellowship (no. 637353). This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NH&MRC IRIISS. A.L. was supported by a JDRF Career Development Award. D.D-A. was supported by an IRO fellowship.

Conflict of interest: The authors declare no financial or commercial conflict of interest. The spouse of A.L. is an employee of UCB Pharma.

References

1 **Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S., Turley, S. J., Von Boehmer, H. et al.**, Projection of an immunological self-shadow within the thymus by the Aire protein. *Science*. 2002. **298**: 1395– 1401.

2 **Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C.**, Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* 2003. **4**: 350–354.

3 **Ahonen, P., Myllarniemi, S., Sipila, I. and Perheentupa, J.**, Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N. Engl. J. Med.* 1990. **322**: 1829–1836.

4 **Peterson, P., Org, T. and Rebane, A.**, Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. *Nat. Rev. Immunol.* 2008. **8**: 948–957.

5 **Peterson, P. and Peltonen, L.**, Autoimmune polyendocrinopathy syndrome type 1 (APS1) and AIRE gene: new views on molecular basis of autoimmunity. *J. Autoimmun.* 2005. **25**(Suppl): 49–55.

6 **Giraud, M., Taubert, R., Vandiedonck, C., Ke, X., Levi-Strauss, M., Pagani, F., Baralle, F. E. et al.**, An IRF8-binding promoter variant and AIRE control CHRNA1 promiscuous expression in thymus. *Nature* 2007. **448**: 934–937.

7 **Vafiadis, P., Bennett, S. T., Todd, J. A., Nadeau, J., Grabs, R., Goodyer, C. G., Wickramasinghe, S. et al.**, Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat. Genet.* 1997. **15**: 289–292.

8 **Kumar, P. G., Laloraya, M., Wang, C. Y., Ruan, Q. G., Davoodi-Semiromi, A., Kao, K. J. and She, J. X.**, The autoimmune regulator (AIRE) is a DNA-binding protein. *J. Biol. Chem.* 2001. **276**: 41357–41364.

9 **Derbinski, J., Gabler, J., Brors, B., Tierling, S., Jonnakuty, S., Hergenhahn, M., Peltonen, L. et al.**, Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J. Exp. Med.* 2005. **202**: 33–45.

10 **Koh, A. S., Kuo, A. J., Park, S. Y., Cheung, P., Abramson, J., Bua, D., Carney, D. et al.**, Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 15878–15883.

11 **Danso-Abeam, D., Humblet-Baron, S., Dooley, J. and Liston, A.**, Models of Aire-dependent gene regulation for thymic negative selection. *Front. Immunol.* 2011. **2**: 14.

12 **Mathis, D. and Benoist, C., Aire**. *Annu. Rev. Immunol.* 2009. **27**: 287–312.

13 **Matsumoto, M.**, Contrasting models for the roles of Aire in the differentiation program of epithelial cells in the thymic medulla. *Eur. J. Immunol.* 2011. **41**: 12–17.

14 **Hubert, F. X., Kinkel, S. A., Davey, G. M., Phipson, B., Mueller, S. N., Liston, A., Proietto, A. I. et al.**, Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood* 2011. **118**: 2462–2472.

15 **Su, M. A., Giang, K., Zumer, K., Jiang, H., Oven, I., Rinn, J. L., Devoss, J. J. et al.**, Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire. *J. Clin. Invest.* 2008. **118**: 1712–1726.

16 **Abramson, J., Giraud, M., Benoist, C. and Mathis, D.**, Aire's partners in the molecular control of immunological tolerance. *Cell* 2010. **140**: 123–135.

17 **Giraud, M., Yoshida, H., Abramson, J., Rahl, P. B., Young, R. A., Mathis, D. and Benoist, C.**, Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells. *Proc. Natl. Acad. Sci. USA* 2011. **109**: 535–540.

18 **Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H. et al.**, Projection of an immunological self shadow within the thymus by the aire protein. *Science* 2002. **298**: 1395–1401.

19 **Fan, Y., Rudert, W. A., Grupillo, M., He, J., Sisino, G. and Trucco, M.**, Thymus-specific deletion of insulin induces autoimmune diabetes. *Embo. J.* 2009. **28**: 2812–2824.

20 **Ohlsson, H., Karlsson, K. and Edlund, T.**, IPF1, a homeodomain-containing transactivator of the insulin gene. *Embo. J.* 1993. **12**: 4251–4259.

21 **Leonard, J., Peers, B., Johnson, T., Ferreri, K., Lee, S. and Montminy, M. R.**, Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. *Mol. Endocrinol.* 1993. **7**: 1275–1283.

22 **Miller, C. P., McGehee, R. E., Jr.and Habener, J. F.**, IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. *Embo. J.* 1994. **13**: 1145–1156.

23 **Gillard, G. O., Dooley, J., Erickson, M., Peltonen, L. and Farr, A. G.**, Aire-dependent alterations in medullary thymic epithelium indicate a role for Aire in thymic epithelial differentiation. *J. Immunol.* 2007. **178**: 3007–3015.

24 **Gordon, J., Xiao, S., Hughes, B., 3rd, Su, D. M., Navarre, S. P., Condie, B. G. and Manley, N. R.**, Specific expression of lacZ and cre recombinase

in fetal thymic epithelial cells by multiplex gene targeting at the Foxn1 locus. *BMC Dev. Biol.* 2007. **7**: 69–81.

25 **Gannon, M., Ables, E. T., Crawford, L., Lowe, D., Offield, M. F., Magnuson, M. A. and Wright, C. V.**, pdx-1 function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. *Dev. Biol.* 2008. **314**: 406–417.

26 **Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L. et al.**, PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996. **122**: 983–995.

27 **Seach, N., Ueno, T., Fletcher, A. L., Lowen, T., Mattesich, M., Engwerda, C. R., Scott, H. S. et al.**, The lymphotoxin pathway regulates Aire-independent expression of ectopic genes and chemokines in thymic stromal cells. *J. Immunol.* 2008. **180**: 5384–5392.

28 **Liston, A., Gray, D. H., Lesage, S., Fletcher, A. L., Wilson, J., Webster, K. E., Scott, H. S. et al.**, Gene dosage–limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J. Exp. Med.* 2004. **200**: 1015–1026.

29 **Ho, W. Y., Cooke, M. P., Goodnow, C. C. and Davis, M. M.**, Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. *J. Exp. Med.* 1994. **179**: 1539–1549.

30 **DiPaolo, R. J. and Unanue, E. R.**, The level of peptide-MHC complex determines the susceptibility to autoimmune diabetes: studies in HEL transgenic mice. *Eur. J. Immunol.* 2001. **31**: 3453–3459.

31 **Liston, A., Lesage, S., Gray, D. H., O'Reilly, L. A., Strasser, A., Fahrer, A. M., Boyd, R. L. et al.**, Generalized resistance to thymic deletion in the NOD mouse; a polygenic trait characterized by defective induction of Bim. *Immunity* 2004. **21**: 817–830.

32 **Aschenbrenner, K., D'Cruz, L. M., Vollmann, E. H., Hinterberger, M., Emmerich, J., Swee, L. K., Rolink, A. et al.**, Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat. Immunol.* 2007. **8**: 351–358.

33 **Villasenor, J., Besse, W., Benoist, C. and Mathis, D.**, Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 15854–15859.

34 **Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K. and Edlund, H.**, beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes Dev.* 1998. **12**: 1763–1768.

35 **Gray, D., Abramson, J., Benoist, C. and Mathis, D.**, Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J. Exp. Med.* 2007. **204**: 2521–2528.

36 **Akkaraju, S., Ho, W. Y., Leong, D., Canaan, K., Davis, M. M. and Goodnow, C. C.**, A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. *Immunity* 1997. **7**: 255–271.

37 **Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. and Papaioannou, V. E.**, RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 1992. **68**: 869–877.

38 **Dooley, J., Erickson, M. and Farr, A. G.**, Alterations of the medullary epithelial compartment in the Aire-deficient thymus: implications for programs of thymic epithelial differentiation. *J. Immunol.* 2008. **181**: 5225–5232.

39 **Gray, D. H., Chidgey, A. P. and Boyd, R. L.**, Analysis of thymic stromal cell populations using flow cytometry. *J. Immunol. Methods* 2002. **260**: 15–28.

40 **Gray, D. H., Fletcher, A. L., Hammett, M., Seach, N., Ueno, T., Young, L. F., Barbuto, J. et al.**, Unbiased analysis, enrichment and purification of thymic stromal cells. *J. Immunol. Methods* 2008. **329**: 56–66.

41 **Livak, K. J. and Schmittgen, T. D.**, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001. **25**: 402–408.

Abbreviations: **Aire**: autoimmune regulator · **cTEC**: Cortical TEC · **Gad1**: glutamate decarboxylase 1 · **Gcg**: glucagon · **HEL**: Hen Egg Lysozyme · **Ins2**: insulin · **insHEL**: Insulin promoter-driven Hen Egg Lysozyme · **mTEC**: Medullary TEC · **Pdx-1**: pancreatic and duodenal homeobox 1 · **Sst**: somatostatin · **TEC**: thymic epithelial cell · **TRA**: tissue-restricted antigens · **T1D**: Type 1 Diabetes

Full correspondence: Dr. James Dooley, VIB – Autoimmune Genetics Laboratory, Campus Gasthuisberg – Department of Experimental Medicine, Herestraat 49, O&N2 bus 1026, B-3000 Leuven, Belgium
Fax: +32-16 330591
e-mail: James.Dooley@vib-kuleuven.be

Additional correspondence: Dr. Daniel H D Gray, Molecular Genetics of Cancer Division, The Walter and Eliza Hall Institute for Medical Research, 1G Royal Parade, Parkville, Australia
e-mail: dgray@wehi.edu.au

Received: 21/6/2012
Revised: 24/8/2012
Accepted: 1/10/2012
Accepted article online: 5/10/2012

