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Update on *Aire* and thymic negative selection

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Summary

Twenty years ago, the autoimmune regulator (*Aire*) gene was associated with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy, and was cloned and sequenced. Its importance goes beyond its abstract link with human autoimmune disease. *Aire* identification opened new perspectives to better understand the molecular basis of central tolerance and self–non-self distinction, the main properties of the immune system. Since 1997, a growing number of immunologists and molecular geneticists have made important discoveries about the function of *Aire*, which is essentially a pleiotropic gene. *Aire* is one of the functional markers in medullary thymic epithelial cells (mTECs), controlling their differentiation and expression of peripheral tissue antigens (PTAs), mTEC–thymocyte adhesion and the expression of microRNAs, among other functions. With *Aire*, the immunological tolerance became even more apparent from the molecular genetics point of view. Currently, mTECs represent the most unusual cells because they express almost the entire functional genome but still maintain their identity. Due to the enormous diversity of PTAs, this uncommon gene expression pattern was termed promiscuous gene expression, the interpretation of which is essentially immunological – i.e. it is related to self-representation in the thymus. Therefore, this knowledge is strongly linked to the negative selection of autoreactive thymocytes. In this update, we focus on the most relevant results of *Aire* as a transcriptional and post-transcriptional controller of PTAs in mTECs, its mechanism of action, and its influence on the negative selection of autoreactive thymocytes as the bases of the induction of central tolerance and prevention of autoimmune diseases.

Keywords: *Aire*; autoimmunity; immune tolerance; negative selection; thymus.

Aire in the time of APECED and its first cloning in 1997

A syndrome with glandular involvement was originally described in 1929[1] that was later termed autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy syndrome (APECED) or autoimmune polyglandular syndrome type 1 (APS1), OMIM entry # 240300. APECED disease is a set of autoimmune manifestations with polyglandular and epithelial compromise, often accompanied by candidiasis. Originally, APECED was characterized as a recessive genetic disease of autosomal transmission with prevalence in the Finnish, Iranian Jewish and Sardinian populations.[2,3] Immunological manifestations are mainly the result of lymphocytic infiltration in the affected organs and the presence of various specific autoantibodies.[3] The defect in the ability of T lymphocytes to react against specific antigens and/or defective immunoregulation could be a cause of the presence of candidiasis in these patients.[3,4]

Genetic analysis showed a link between APECED and mutations on human chromosome 21, 21q22.3, a region that had not previously been associated with autoimmune diseases.[5] Genetic studies allowed the identification of a new gene whose structural characteristics, such as two PHD motifs, suggested its role as a transcriptional regulator. The mutations present in this gene and associated with the presence of APECED allowed the establishment of a strong relationship between the disease and that gene, which was then denominated Autoimmune

regulator (*Aire*) because of its possible role in the prevention of aggressive autoimmunity.[3,5,6] In 2017, we celebrate 20 years of *Aire* cloning and sequencing. Of note, this gene was the first to be located outside the human MHC locus that had been associated with autoimmunity (OMIM # 240300).

However, it was not feasible to study this question easily in humans, so the role of *Aire* in the negative selection of autoreactive thymocytes was only demonstrated with the use of the mouse model.

Function of *Aire*

The human autoimmune regulator (*Aire*) gene is 11·9 kb long, spanning 14 exons, and encodes a 1635-nucleotide mRNA, which in turn encodes a 545-amino-acid protein. In the mouse, the *Aire* gene is located on chromosome 10 position 39·72 cM, is 13 kb long, and encodes a 14-exon mRNA whose encoded protein has 545 amino acid residues with 77% human–mouse identity.[7] (Fig. 1).

Expression of the *Aire* gene is preferentially observed in the thymus, pancreas, adrenal cortex and testes;[3,8] however, it was also observed in lymph nodes, fetal liver and appendix.[6] To better understand the effect of mutations in the *Aire* gene on its biological function and manifestations in APECED, it is important to understand its primary structure, which suggests the typical domain distribution of a protein capable of interacting with the chromatin and regulating transcriptional processes.[9] The human AIRE protein has a molecular weight of 56 KDa with five subdomains: the CARD domain (caspase recruiter domain) that is important in the oligomerization of the protein; the Nuclear Location Signal that mediates its migration from the cytoplasm to the nucleus; the SAND domain (SP100, AIRE1, NucP41/P75 and DEAF1) involved in promoting protein–protein interactions; and two PHD domains, PHD-1 and PHD-2, that function as histone code readers, interacting and aiding in the chromatin decondensation.[10–12]

The inheritance of human *Aire* mutations is preferentially recessive and is usually accompanied by severe clinical manifestations of the disease.[2] However, new dominant variants have also been described in different domains of the *Aire* gene, but these are usually accompanied by detectable intermediate or detectable auto-antibodies, which would indicate nothing more than disease susceptibility.[13] Consequently, each domain has a special function both in the regulation of the protein and in its transcriptional effects on the cell; additionally, depending on where the mutation occurs, *Aire* is affected in many ways.[10,13]

In an *Aire* knockout (Aire$^{-/-}$ KO) murine model, the mice present changes in their thymic structure, deficiencies in the expression of peripheral tissue antigens (PTAs) in medullary thymic epithelial cells (mTECs), the presence of specific autoantibodies against self-antigens, the decrease in regulatory T cells and the activation of self-reactive T cells, promoting a general autoimmune reaction similar to that found in APECED patients. In this case, the mutation was generated in embryonic stem cells, leading to a deletion of the *Aire* exon 2 and resulting in a truncated protein with loss of the SAND and PHD-1 and -2 domains.[14,15]

Aire as a pleiotropic gene

As AIRE protein acts based on transcriptional control in mTECs and nonspecifically, several downstream genes
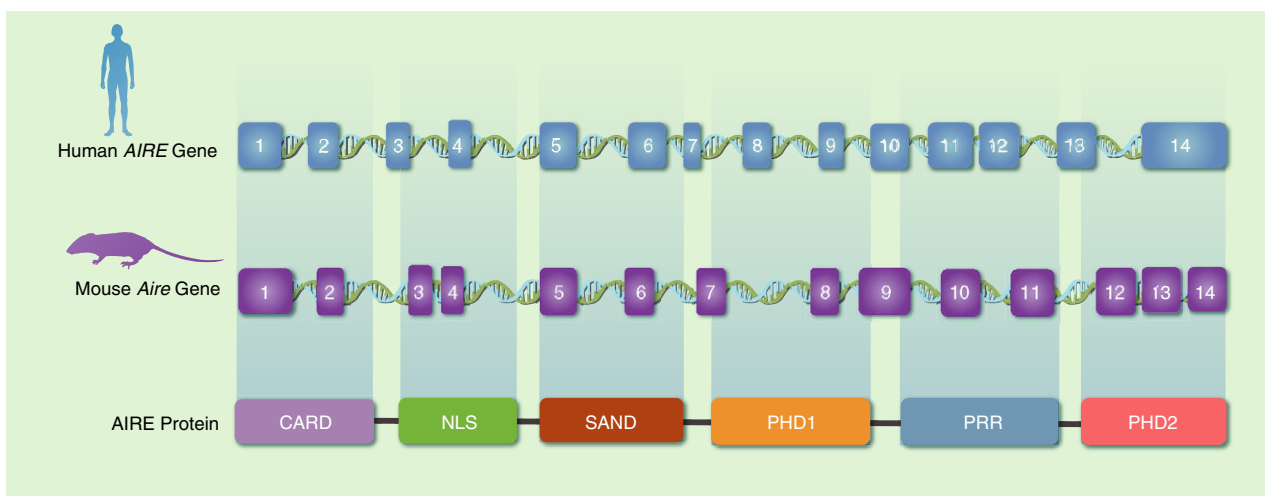

Figure 1. Schematic representation of the *Aire* gene and its respective encoded protein. Both human (chromosome 21q22.3) and mouse (chromosome 10 position 39·72 cM) *Aire* spans 14 exons and encodes AIRE protein with six domains.

and biological functions are subjected to its control; therefore, the *Aire* gene may be considered pleiotropic, and its different known functions are presented below.

Functional marker of mTEC differentiation

The expression of *Aire* is restricted to a few organs, among them the thymus. However, not all cells that integrate the thymic microenvironment can express this gene and its respective protein.[3] Within the thymus, only mTECs can express this gene. In addition, not all mTECs express *Aire* at any given time, the expression of which appears to be limited to a small subpopulation of mature mTECs whose phenotype is CD80^{hi} MHC-II^{hi}.[16–20]

It has been demonstrated that the expression of *Aire* is an inherent property of mTECs during their differentiation programme and may occur at specific stages and/or cell states.[21] *Aire* expression can be modulated by epigenetic factors, such as methylation,[22,23] or by molecular mechanisms, such as the induction of the nuclear factor-κB pathway through RANKL–RANK-mediated signalling, CD40L–CD40 or the leukotriene β-mediated pathway, that determine the fate of the differential expression of *Aire* only in a subset of mTEC cells.[24–26] Whether *Aire* might be post-transcriptionally controlled through microRNAs (miRNAs) remains an open question.[27]

Populations of CD80^{low} MHC-II^{low} mTECs, which do not express the *Aire* gene, not only present a significant reduction in the expression of PTAs but also greater proliferation and may also indicate a role of *Aire* in the control of proliferation and maintenance of the thymic structure through mechanisms of apoptosis.[10,15,18,19]

Controlling the expression of peripheral tissue antigens

The murine genome has approximately 24 000 protein-coding genes described so far, of which mTEC^{hi} cells express approximately 15 000 genes, which represent more than 62% of the mouse genome.[18] The mTECs can express a broad number of tissue-specific genes, a phenomenon that has not been observed in any other type of epithelial cell. This makes mTECs the most peculiar yet identified given the diversity of expressed genes and proteins. This ectopic gene expression appears to be a unique feature of the thymus and does not correspond to a defect of cell transcriptional control but rather to an inherent ability of these cells to promote promiscuous gene expression (PGE).[23,27–30]

Irrespective of their role in the periphery, self-regulatory T (Treg) cells are negatively selected during the induction of central tolerance in the thymus. The thymic microenvironment is composed of different sets of antigen-presenting cells, such as cortical TECs (cTECs), mTECs and thymic dendritic cells [(DCs) which present

only PTAs that are expressed and processed by mTECs].[29,31–40]

Aire regulates the expression of more than 3200 downstream genes in mTECs through a large transcriptional complex.[10,18,41,42] It is important to understand that *Aire* is not a conventional transcription factor because it does not bind directly to any DNA motifs of PTA genes. Rather, AIRE protein interacts and releases RNA Pol II molecules that have already started transcription but are stalled at the promoter regions of the genes – in other words, AIRE pushes them to proceed with gene transcription.[43] In our view, this finding ultimately contributed to a better understanding of *Aire*'s promiscuous action – that is, its protein favours the transcriptional activity of RNA Pol II, which is not specific and covers most, if not all, of the protein-coding genes of a mammalian cell. Due to its vast heterogeneity, this type of expression was called PGE. It is important to note that the biological meaning of PGE is immunological – i.e., the ectopic proteins expressed in the thymus play their role as PTAs that are presented in the form of peptides and via MHC-II to the developing thymocytes during the negative selection process.[28]

How *Aire* temporally determines the transcription of these genes is subject to extensive study. The expression of *Aire*-dependent PTAs in mTECs was interpreted as stochastic and at the same time 'ordered'. The term 'stochastic' was used because only a small percentage (1–3%) of mTECs express a certain PTA at any given time, although *Aire* promotes the expression of several PTAs in one mTEC at the same time. More than co-expression, this seems to be a probabilistic event. The term 'ordered' refers to an increased likelihood of a particular set of PTA genes to be often co-expressed in a single mTEC. *Aire* could determine, in an orderly way, the expression of genes that are within the same chromosomal region or forming networks between chromosomes by synchronizing their expression.[10,44–46]

In fact, the AIRE protein is associated with a broad set of proteins to initiate the transcription of PTAs in mTECs that can be classified into four functional groups: proteins involved in nuclear transport, proteins that interact directly with chromatin, and proteins that can participate in both transcription and mRNA processing (Table 1). Additionally, there are proteins that, despite not interacting with AIRE, participate in the transcriptional complex associated with the expression of PTAs in mTECs.[10] Many of these molecules even regulate *Aire* itself.[47]

Recently, the group of Benoist/Mathis at Harvard University in Boston, MA, have found that some AIRE partners, especially those associated with the DNA-damage response, localize preferentially on chromatin sections known as super-enhancers, as for example TOPO 1.[48]

Table 1. AIRE partners during the promiscuous gene expression in medullary thymic epithelial cells[23,43,91–109]

Protein symbol	Name	Function	AIRE domain involved
Proteins that interact concomitantly with AIRE protein			
RNA Pol II	RNA Polymerase II	Starts the gene transcription after *Aire*-partners-mediated activation and phosphorylation	PHD-2
H3K4	Unmethylated histone H3 lysine 4	Recruits AIRE protein at the transcription start site(s) (TSS)	PHD-1
ATF7IP–MBD1	Activating transcription factor 7-interacting protein–methyl-CpG-binding domain protein 1	Mediates AIRE protein recruitment by methylation of CpG island in repressed genes	SAND
DNA-PK	DNA-dependent protein kinase	Phosphorylates the AIRE protein, indicates where *Aire* starts the gene transcription by phosphorylation of H2AX histone that recognize DNA breaks	PHD-2
P-TEFb	Positive transcription elongation factor b	Promotes that AIRE protein unleashes stalled RNA Pol II	C-Terminus
HNRNPL	Heterogeneous nuclear ribonucleoprotein L	Participates in the RNA elongation mediated by pTEFb	C-Terminus
BRD4	Bromodomain-containing 4	Binds directly to AIRE protein and forms a complex with pTEFb and HNRNPL to induce the unleashes of stalled RNA Pol II	CARD
EFTUD2	Elongation factor TU GTP-binding domain-containing protein 2	Involved in the alternative splicing of RNA mediated by AIRE protein	ND
SNRPB	Small nuclear ribonucleoprotein-associated proteins B		
SRSF1	Serine/arginine-rich splicing factor 1		
SIRT1	Deacetylase sirtuin 1	Deacetylates lysine residues of AIRE protein leading to its activation	Lysine residues NLS (121–261)
HDAC1-2	Histone deacetylase 1 and 2	Exports and stabilizes AIRE protein in the nucleus	
CBP, p300	CREB-binding protein	Acetylates the lysine residues of AIRE protein leading to repression of its activity	
HIPK2	Homeodomain-interacting protein kinase 2	Phosphorylates AIRE protein suppressing its activation	PHD-2
FBXO3	F-box protein 3 E3 ubiquitin ligase	Ubiquitinates AIRE protein promoting and increasing its affinity to pTEFb factor	N terminus (207)
MARCH 8	Membrane-bound E3 ubiquitin ligases	Regulates the MHC-II expression in AIRE+ medullary thymic epithelial cell (mTECs)	ND
PIAS1	Protein inhibitor of activated STAT	Possible sumoylation of AIRE protein regulating its activation	CARD
Importins	Importins α1, α3 and α5	Exporting of AIRE protein to the nucleus	NLS
TOP2A	DNA topoisomerase IIα	Assiciates with AIRE protein and promotes DNA breaks at TSS	PHD-2
Proteins that directly interact with the *Aire* gene (at DNA level)			
ERα	Oestrogen receptor α	Induces methylation of the promoter region of *Aire* gene leading to decreasing of *Aire* expression	Promoter
AR	Androgen receptor	The accumulation of AR in the promoter region of Aire gene leads to its activation	Promoter
JMJD6	Histone arginine demethylase	Promotes the splicing of intron 2 of *Aire* mRNA	Intron 2
CNS1	Conserved non-coding sequence 1	Conserved region of 90 nucleotides that regulate the *Aire* gene activation through RANK and nuclear factor-κB signalling	Between *Aire* and Dnmt3l genes
IRF4, IRF8, TBX21 and TCF7	Interferon regulatory factor 1 e 8, T-box transcription factor, transcription factor 7	Form a transcriptional complex necessary for the *Aire* gene expression in mTECs	Promoter
CTCF	CCCTC-binding factor	Represses the *Aire* gene expression inhibiting the interaction of IRF4, IRF8, TBX21 and TCF7 complex with the *Aire* promoter	TSS and 3′ UTR

In summary, *Aire* leads to the activation of PGE in mTECs when it is recruited into the transcriptional start sites (TSS) of most genes. Once joining stalled RNA Pol II and through histone modifications and deacetylation mediated by SIRT-1, the complex allows TOP2a to generate disruptions in the regions of transcription initiation that induce epigenetic changes and relaxation of the chromatin. In turn, this allows the recruitment of the pTEFb/BRD4 complex, releasing RNA Pol II and subsequent transcription elongation.[49] All these elements working in a cascade show not only how AIRE protein controls PGE but also how the *Aire* gene is controlled (Fig. 2, Table 1).

Role of *Aire* beyond the control of PTA expression

The role of *Aire* in the expression of PTAs and in the prevention of the development of aggressive autoimmunity has been widely studied and debated. Nevertheless, whether *Aire* participates in other processes besides the negative selection of T cells remains a topic of research.[10] It has been reported that *Aire* also participates in mechanisms of B-cell tolerance, an event that does not occur in the thymus. Patients with APECED develop highly specific autoantibodies against a group of c.100 self-proteins. However, some of these antibodies are neutralizing and have the function of decreasing the pathological effects of pro-inflammatory cytokines. This could explain why APECED patients do not show symptoms of cytokine-mediated diseases such as the interferon-γ seen in systemic lupus erythematosus or systemic sclerosis.[50] *Aire* also regulates the expression of genes that are not listed as autoantigens;[18] many of these genes are involved, for example, in the recruitment of DCs into the thymus medulla, as in the case of the gene that encodes XCL1 ligand that binds to the XCR1 chemokine receptor.[51] *Aire* could also influence the recruitment of cells in the thymic microenvironment through the induction of chemokine expression.

On the other hand, studies carried out independently in our group at the University of São Paulo in Ribeirão Preto, Brazil and in the Kyewski/Ucar group at the German Cancer Research Centre in Heidelberg, Germany, demonstrated the importance of *Aire* in the expression of miRNAs. Partial reduction of the *Aire* gene leads to a change in the miRNA expression of mTECs.[52] Therefore, miRNAs could be important in regulating PGE in mTECs under the influence of *Aire*; consequently, miRNAs could be important in the organization of thymic architecture.[23,27,53]

Aire can also regulate mRNA alternative processing, possibly allowing mTECs to increase their repertoire of PTAs. Aire$^+$ CD80^{hi} MHC-II^{hi} mTECs have an alternative splicing rate higher than that of other cell populations.[18,54,55]

Aire controlling mTEC-thymocyte adhesion

In the thymic microenvironment, the AIRE$^+$ and AIRE$^-$ mTECs, DCs and thymocytes continuously interact in the medulla, contributing to the central tolerance of the T cells to self-antigens.[56] The mTECs using *Aire* control do not only express PTAs but also molecules involved in cell–cell interaction processes and some important adhesion molecules.[18] In turn, the expression of these genes is dependent on the levels of the AIRE protein, so that studies using the small interfering RNA (siRNA) technique allowed the demonstration that the reduction of *Aire* leads to a concomitant reduction not only in PTAs but also in the adhesion capacity of mTECs to thymocytes.[57] The molecules most affected by the reduction in the *Aire* levels provoked by siRNA are integrins, claudins and adhesion or co-stimulatory molecules as CD80.[57] These findings were of great importance because mTEC–thymocyte adhesion is a crucial process for the negative selection of autoreactive thymocytes and induction of immunological tolerance.

Aire and apoptosis of mTECs

A controversial function of *Aire* is its possible role in the induction of apoptosis in mTECs.[19] Due to the increase in MHC-II^{hi} mTECs under *Aire* deficiency, a possible explanation could be that *Aire* promotes apoptosis in these cells. This stimulation supposes that apoptotic cells could be phagocytosed and PTAs could be presented through antigen-presenting cells, increasing self-representation.[34] Nevertheless, this function was not yet confirmed due to conflicting observations from an inducible Cre mouse model, whose results indicated that *Aire* is not correlated with the mTEC lifespan.[58]

Promiscuous gene expression added a second controller: *Fezf2*

According to Sansom *et al.*,[59] murine thymic epithelial cell populations express more than 19 000 protein-coding genes, which confer the set of PTAs and other non-PTA genes. Currently, no other type of cell has shown this enormous transcriptional diversity. In the mouse and this set of genes, *Aire* itself controls 3900 genes, which are considered *Aire*-dependent, that correspond to approximately 20% of PGE. No doubt *Aire* is an important transcriptional controller, but other controllers should still work independently resulting in PGE. In fact, this is a long-standing suspicion because PGE was not completely aborted under *Aire* annulment.[60]

As elegantly discussed by Klein,[61] over the years, researchers have devoted their efforts to elucidating the mechanism by which *Aire* works; however, the regulation
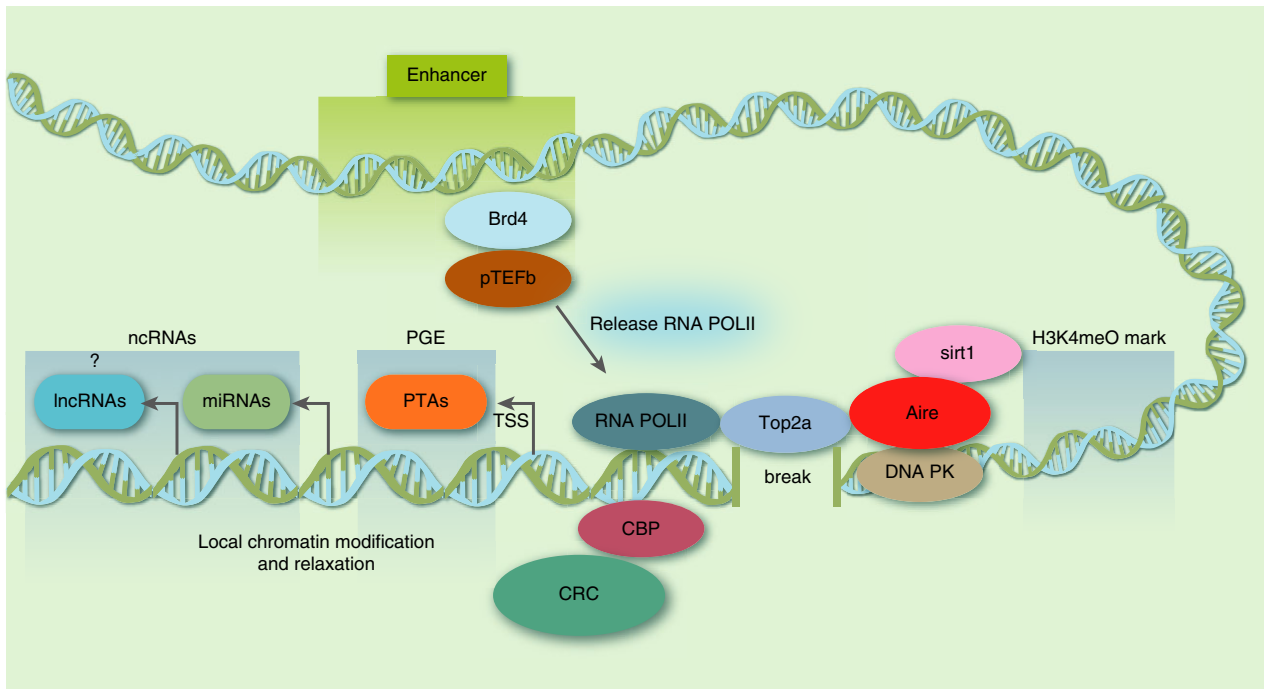
Figure 2. Mechanism of AIRE and its partners. AIRE protein binds to the chromatin in association with several other proteins. In this partial view, the protein complex binds to the transcription start site (TSS) of genes in medullary thymic epithelial cells (mTECs). Deacetylase sirtuin 1 (SIRT-1) deacetylates lysine residues in AIRE leading to its activation. Then, AIRE through its PHD1 domain binds to AIRE-dependent genes recognizing repressive epigenetic signatures that includes H3K4me0. TOP2a at the TSSs of AIRE-dependent genes initiates breaks on DNA. DNA-PK and other partners are activated by the DNA breaks resulting in local chromatin relaxation. The pTEFb/BRD4 complex releases stalled RNA POL II on chromatin, causing ensuing transcription elongation of AIRE-dependent genes, including peripheral tissue antigen (PTA) -encoding genes and non-PTA genes. As microRNAs are transcribed by RNA POL II, this species of non-coding RNAs is regulated by AIRE in mTECs. The long non-coding RNAs (lncRNAs), also transcribed by RNA POL II, could, perhaps, be regulated by AIRE? Finally, CREB-binding protein (CBP) acetylates the lysine residues in AIRE leading to its repression. This figure is a modification of figure 2 published by Abramson J and Husebye ES. *Immunol Rev* 2016;271:127 (with permission of John Wiley and Sons).

of *Aire*-independent genes had remained elusive. Major progress occurred with the appearance in the literature of the work of Takaba *et al.*,[62] in which the authors considered that, if there was a transcriptional regulator of *Aire*-independent genes, it should be differentially expressed between cTECs and mTECs. In fact, these authors identified the forebrain-expressed zinc finger 2 (*Fezf2*) that is expressed in mTECs but not in cTECs. *Fezf2* had already been associated with corticospinal motor neuron differentiation and, as *Fezf2*-deficient mice do not survive after weaning, the role of this gene in the immune system remained undetected.

However, when examining *Fezf2*-deficient cells, a set of PTAs are down-regulated that do not coincide with those down-regulated in *Aire*-deficient mTECs. This clearly demonstrated that *Fezf2* and *Aire* work independently but synergistically in the control of PGE in mTECs. Much more rapidly, with the elucidation of the mode of action of *Aire* in chromatin, Takaba's work demonstrated that, in contrast to AIRE, FEZF2 protein directly binds to DNA near its target genes like a classical transcription factor.

Takaba's work on *Fezf2*, besides contributing to a better understanding of the PGE and molecular bases of central tolerance, stimulates new issues such as its role (i) in the organization of the three-dimensional thymic structure,[61] (ii) as an upstream controller of non-PTA protein-coding genes (chemokine or cell adhesion genes, for example), (iii) as an upstream controller of non-coding RNAs [miRNAs and long non-coding RNAs (lncRNAs)] and (iv) as a controller of mTEC–thymocyte adhesion, among other questions that are sure to come.

T-cell differentiation, negative selection and central tolerance

As mentioned above, the thymus is the anatomical site where central tolerance occurs. This organ is divided into two main distinct regions, the cortex and medulla, and each provides a complex set of signals that will allow bone-marrow-derived progenitor cells to develop into the organ and finally become mature T cells. These cells, which were rescued from programmed cell death, acquire the ability to exit the organ and migrate to the

thymus-dependent areas of the secondary lymphoid organs.[36,63]

Among the cells that compose the thymus stroma, such as DCs, macrophages, fibroblasts, B cells and others, TECs are the major component. They are located in both the cortex (cTECs) and medulla (mTECs) of the thymic lobules and have distinct phenotypes, secretomes and roles in thymocyte maturation and selection processes.[64,65] TECs produce components of the thymus microenvironment, such as insoluble extracellular matrix (ECM) molecules and soluble components like interleukins, growth factors and chemokines that mediate ECM–cell and cell–cell interactions, respectively.[66]

In the immune system, particularly in the thymus, cell–cell interactions are importantly mediated by T-cell receptor (TCR)/peptide–MHC signalling, which, in combination with various other stimuli, define cell activation, proliferation and survival. In the cortex, immature double-negative ($CD4^-$ $CD8^-$) and double-positive ($CD4^+$ $CD8^+$) thymocytes interact with cTECs, in addition to fibroblasts and macrophages that are also present.[67] At this point, TCR-β rearrangement is tested through the expression of a pre-TCR in a process called β-selection, and failure to generate a functional receptor results in apoptosis. Following the TCR-α chain rearrangement, the assembly and expression of the definitive TCR-$\alpha\beta$ at the highly expanded double-positive stage and TCR specificity are tested in the process known as positive selection. Here, low-avidity interactions with self-peptide–MHC complexes expressed by cTECs allow the survival and further differentiation of thymocytes. Next, thymocytes start losing the expression of either CD4 or CD8 and migrate from the cortex through the corticomedullary junction, reaching the thymic medulla.[68]

The medullary region is relatively poor in thymocytes and abundant in mTECs, which play a central role in thymocyte negative selection. Macrophages, B cells, resident and migratory dendritic cells (DCs) are also found in the medulla and can contribute to this process, as discussed below. The pathways leading to CD4 or CD8 single-positive ($CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$) subsets are dependent on antigen presentation by TCR interaction with peptide–MHC class II or class I molecules, respectively, and distinct transcriptional machineries induced also by other cell surface and soluble molecules, such as Delta-class Notch ligand Delta-like 4 (Dll4), kit ligand and interleukin-7.[36] High-affinity TCR interactions with MHC self-antigens induce the deletion of thymocytes by apoptosis or skew to alternative fates such as the differentiation of Treg cells ($CD4^+$ $CD25^+$ $FOXP3^+$), so contributing to the establishment of a T-cell repertoire that can respond to non-self-antigens and is tolerant to self-antigens. The particular mTEC $CD80^{hi}$ $MHC-II^{hi}$ subset that expresses *Aire* and can present PTAs is crucial for negative selection.[69] These processes of positive and negative selection are the mechanisms of the generation of central tolerance that prevent aggressive autoimmunity (Fig. 3).

Although the role of *Aire* is well defined in thymocyte negative selection, its role in Treg cell differentiation and selection remains controversial.[49,70] The generation of certain Treg clones is dependent on *Aire* expression. As an example, $FOXP3^+$ MJ23tg and RT83tg Treg cells, which are found infiltrated in the tumours of mice with oncogene-driven prostate cancer, failed to develop in the thymus and $Aire^{-/-}$ hosts.[71] Other works have suggested that Treg cell differentiation is not dependent on *Aire* but on PTA–MHC presentation by other bone-marrow-derived antigen-presenting cells such as DCs. Interestingly, because *Aire* is not expressed in these antigen-presenting cells, it was suggested that mTEC antigens could be transferred to DCs.[72,73]

Intrathymic T-cell differentiation, including positive and negative selection processes, occur concomitantly with highly regulated migratory events, from the entrance of the progenitors until the exit of mature single-positive cells to the periphery, a process that allows developing cells to interact with the different niches of the thymus. In this context, it was shown that $Aire^{-/-}$ mice show aberrant expression of migration-related molecules, such as CCR4 and CCR7 ligands. Consequently, the $Aire^{-/-}$ thymus presented a delay in CD4 single-positive cell emigration as early as 5 days after birth.[74] Recent work extended these analyses in neonatal mice and showed that the $Aire^{-/-}$ thymus revealed significantly lower levels of CCL22 (CCR4 ligand) and CCL19 (CCR7 ligand) as well as CCL5 (ligand for CCR1, 3, 4 and 5), CCL17 (ligand for CCR8) until 6 weeks of age. Interestingly, reduced thymocyte emigration only occurred within the first 3 weeks after birth and was associated with the decreased expression of the CCR7 ligands. Conversely, an early and transient up-regulation of sphingosine-1-phosphate receptor 1 expression was observed in $Aire^{-/-}$ thymocytes.[75] Because sphingosine-1-phosphate receptor 1 is essential for thymocyte egress,[76] one can argue that compensation mechanisms occur, allowing thymocyte emigration during this period.[76] Together, these data show that the absence of *Aire* can also disturb peripheral tolerance by reducing the emigration of mature SP cells. Whether reduced emigration of Treg cells is involved must be investigated.

As discussed above, the absence of *Aire* in mTECs *in vitro* modulates various genes encoding proteins involved in cell adhesion and migration such as laminin, intercellular adhesion molecule 1 and vascular cell adhesion molecule 4. Functionally, *Aire* deficiency reduced the adhesion of thymocytes to mTECs that can modulate thymocyte migration and differentiation, contributing to abnormal tolerance or the breakdown of tolerance.[57]
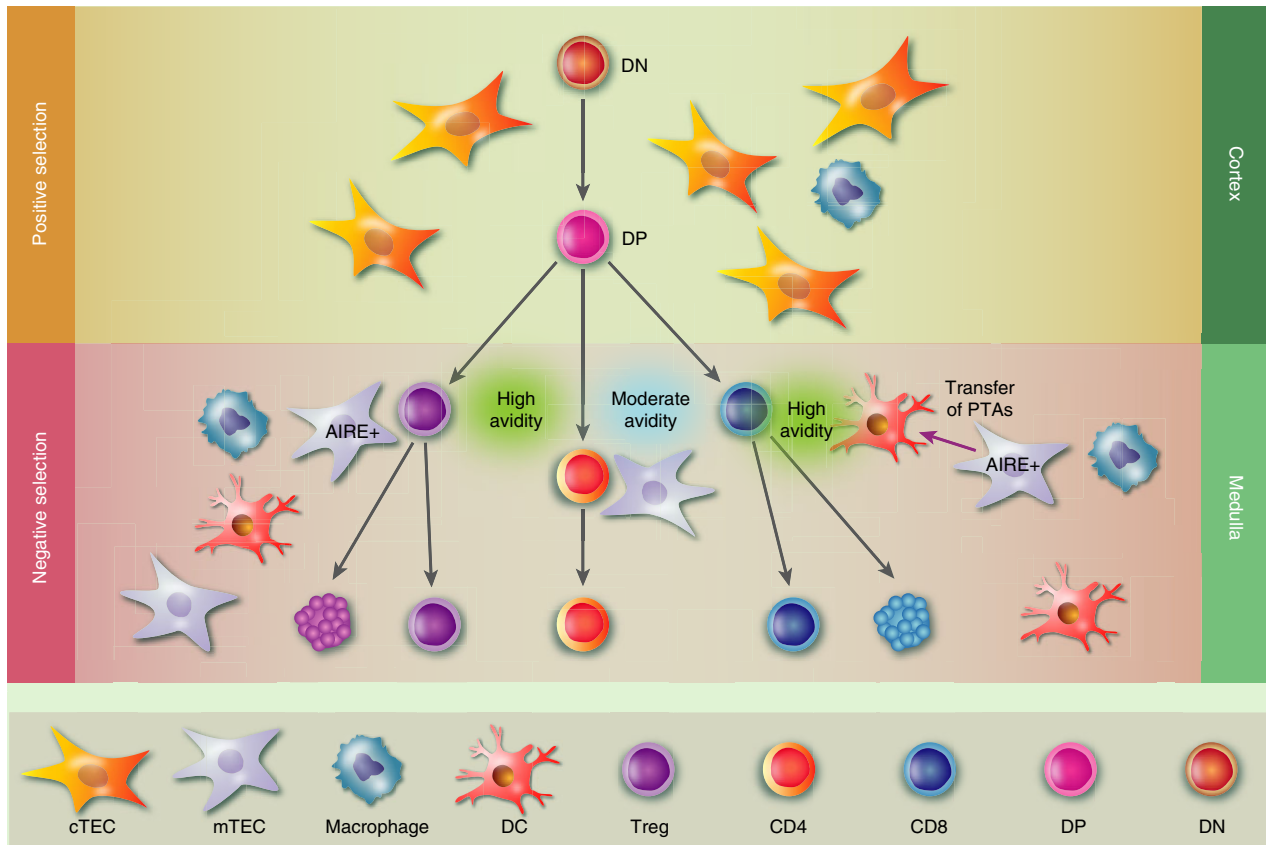
Figure 3. Medullary thymic epithelial cell (mTEC) –thymocyte interactions and negative selection. Negative selection occurs in the thymus in the medullary region. Double-positive (DP; CD4$^+$ CD8$^+$) thymocytes from the inner cortex start losing the expression of either CD4 or CD8 and migrate from the cortex to the thymic medulla where they interact with mTECs and other bone-marrow-derived antigen-presenting cells, such as dendritic cells. High-affinity T-cell receptor interactions with self-antigens presented by MHC molecules induce the deletion of thymocytes by apoptosis or differentiation for regulatory T cells (CD4$^+$ CD25$^+$ FoxP3$^+$ Treg cells). The particular AIRE$^+$ mTEC subtype capable of presenting peripheral tissue antigens (PTAs) is crucial for this process. AIRE$^+$ mTECs can also transfer PTAs for dendritic cells that will also play a role in negative selection. Moderate-avidity T-cell receptor interactions with self-peptide and MHC class I or II complexes expressed by cortical TECs allow survival and further differentiation of thymocytes to CD4 or CD8 single-positive subsets (CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$) respectively, that will leave the thymus to the peripheral lymphoid organs.

In addition to the indirect control of adhesion, migration and egress of thymocytes, *Aire* can modulate DC migration and localization though the expression of XCL1. *Aire*$^{-/-}$ mice showed diminished mTEC expression of XCL1 and DC accumulation in the medulla, which could, in turn, disturb antigen transfer between mTEC and DCs.[51] Interestingly, both *Aire*- and XCL1-deficient mice presented low numbers of Treg cells. In this case, the authors hypothesized that *Aire* is essential for the mTEC expression of XCL1, which attracts XCR1-expressing DCs to the medullary region and contributes to the optimal generation of Treg cells. Together, these studies show that *Aire* can directly regulate negative selection by controlling gene expression and, indirectly, by modulating the expression of molecules that play a role in cell–cell adhesion and cell migration.

Association between *Aire* gene mutations with loss of self-tolerance in humans

More than 110 mutations along the *Aire* gene have been identified and associated with APECED or other autoimmune diseases in humans. Some populations present a major genetic association with a particular mutation being p.R257*, which is located within the SAND domain, the most common.[9] Although APECED is a monogenic disease, it has a diverse range of clinical manifestations, often without a strict genotype–phenotype association. This suggests that not only the genetic risk, but the genetic mutation *per se* may be acting in immunological dysregulation and loss of self-tolerance; environmental factors,[70] or even imbalance in the thymic gene expression, might play a role in this pathogenic process.

Patients with APECED present in both children and adults with a significant decrease of CD4^{+} CD25^{+} Foxp3^{+} Treg cells,[70] failure in thymic selection of naive T cells with defects in TCR repertoire[77] or abnormal expression of cytokines.[78]

In addition, B cells that differentiate within the thymus express *Aire*[79,80] and APECED patients present an altered B-cell phenotype and dysregulation in B-cell function with B-cell reactivity to a unique set of approximately 100 self-proteins and production of neutralizing autoantibodies against specific cytokines.[81,82]

Despite the broad diversity of PTAs expressed by mTECs through PGE, patients with APECED present reactivity only against a minor fraction of self-proteins,[83] indicating that *Aire* may be acting beyond the selection of T cells.

Depending on the localization of mutations along the human *Aire* gene and the AIRE protein domain affected, the clinical manifestation can be different. For example, dominant mutations in the PHD1–2 and SAND domain affect the protein structure and correlate with the presence of organ-specific autoimmune diseases.[84] In addition, mutations in the promoter region impair the transcriptional activity of *Aire*[85] or mutations in the CARD/HSR domain affect the localization and export of AIRE protein from cytoplasm to the nucleus and mutations in intronic regions can alter the splicing of *Aire* mRNA.[84,86,87]

Summary

The current update on the molecular control of central immune tolerance that ultimately leads to negative selection in the thymus supports the participation of the *Aire* gene/protein and its partners. These molecular elements form a complex that finally binds to stalled RNA Pol II on the chromatin of mTECs, promoting the enzyme to proceed the transcription elongation phase of PTAs, as well as that of other non-PTA genes. Because RNA Pol II is non-specific and binds to the transcription start sequences of genes, this could clarify how the *Aire* gene controls PGE. In addition, recent evidence strongly suggests that *Aire* may play a role as an upstream controller of post-transcriptional processes involving miRNA–mRNA interaction in mTECs. Many genes encoding PTAs in mTECs do not undergo *Aire* influence and are under the effect of the *Fezf2* gene/protein that directly binds to a large set of transcription start sequences in the chromatin of these cells. With the recent association of the *Fezf2* gene/protein in the control of PGE, other questions open in the light of what we have learned from *Aire* over the past 20 years. Are there *Fezf2* DNA polymorphisms that could be associated with the loss of immune tolerance or emergence of autoimmune diseases? Are the genes associated with cell adhesion

controlled by *Fezf2*? What are the epigenetic factors associated with the function of *Fezf2*? Does *Fezf2* control non-coding RNAs (miRNAs and/or lncRNAs)? Finally, what are other upstream controllers besides *Aire* and *Fezf2* that regulate PGE in mTECs? There are two other questions still not fully resolved about the role of Aire *in vivo*; one of them is on the possible influence of variations of the levels of expression of *Aire* related to ageing thymus in the onset of aggressive autoimmunity.[88] Considering the location of the *Aire* gene on human chromosome 21q22.3, the other question regards the effect of *Aire* gene dosage in children with Down syndrome.[89] Finally, a new perspective was pointed out by the group of Nuno Alves at the Thymus Development and Function Laboratory (IBMC), Porto, Portugal, that communicated the participation of p53 during mTEC differentiation. Loss of p53 disrupts the integrity of the niche of mTECs while influencing the cTEC compartment.[90]

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Disclosure

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