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PERSPECTIVES

TIMELINE

A decade of AIRE

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Abstract | In 1997, the autoimmune regulator (*AIRE*) gene was identified as the locus underlying susceptibility to the polyendocrine autoimmune disease known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). In the intervening 10 years, it has become increasingly clear that this rare disorder has provided us with an illuminative window on one of the most fundamental processes of the immune system — the establishment and maintenance of self tolerance.

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; also known as APS1) is characterized by a set of three abnormal features — chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency[1] — a clinical triad first reported in 1946 (REF. 2) (TIMELINE). Classically, an individual must present with at least two of these three abnormalities to be diagnosed with APECED, which usually happens before the age of ten. Patients with APECED also routinely exhibit a variable number of other autoimmune manifestations, including thyroiditis, type 1 diabetes, ovarian failure and hepatitis. These secondary features differ widely from patient to patient, even between siblings with exactly the same genetic lesion and similar environmental exposures.

APECED was highlighted as having unusually simple genetics for an autoimmune disease[3]. Exhibiting an autosomal recessive mode of inheritance, it was generally considered to be a monogenic disorder. However, more recent analyses have revealed influences of additional genetic loci, in particular the HLA complex, on certain disease parameters, such as the development of type 1 diabetes[4,5]. APECED is a rare disorder, with pockets of elevated frequency in Finland, Sardinia and Iran[6].

A major leap forward in our understanding of APECED came in 1997 when two groups used heroic positional cloning strategies to identify the underlying gene[7,8]. The protein encoded at this locus was termed AIRE (autoimmune regulator). It is a large protein of 545 amino acids with several domains reminiscent of those found in transcriptional regulators, notably zinc-finger-containing PHD motifs and a SAND domain (named after Sp100, AIRE1, NucP41/75 and DEAF1/suppressin), which serves as a DNA-binding element in some transcription factors but is missing the critical DNA-interacting residues in AIRE[9]. Over 60 mutations have been localized in the *AIRE* gene of different patients with APECED. Given that the mutations are scattered throughout the protein sequence, few insights into the function of AIRE have been gained from studying them; in addition, these different mutations have not, to date, been convincingly associated with particular disease manifestations. At the time of its discovery, there was significant controversy concerning the pattern of AIRE expression, with some groups claiming a very broad organ distribution[7], and others a distribution quite restricted to lymphoid organs, in particular the thymus[8,10]. This discrepancy no doubt reflected differences in probe specificity and technique reliability and were eventually resolved in favour of the latter view[11].

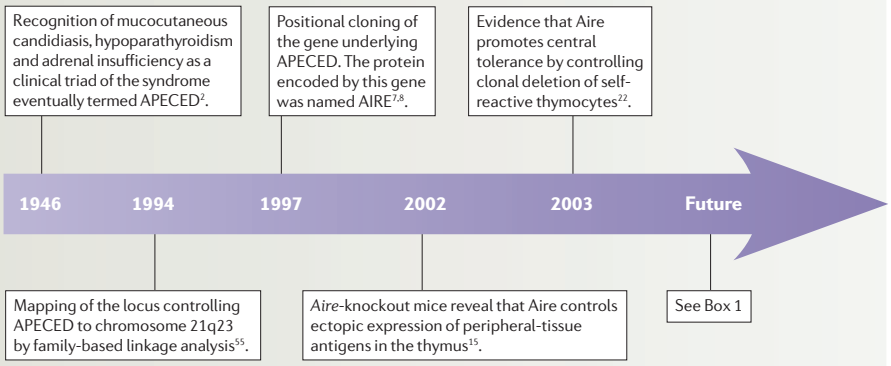
Knowledge of the structure and expression of AIRE prompted a number of early hypotheses on how it might operate to control autoimmunity. These hypotheses include: determining the organization of the thymic stroma[12]; controlling thymocyte tolerization[8]; regulating B-cell and T-cell responses to antigenic stimuli[7]; inducing apoptosis of parenchymal cells and thereby enhancing cross-presentation of their antigens[13]; or impinging on the differentiation of $CD4^+CD25^+$ T regulatory (T_{Reg}) cells[13]. Given the impossibility of evaluating such ideas in humans, investigators rapidly cloned mouse *Aire* in order to develop an experimental model of APECED[14].

Major mechanisms of Aire in mice

Studies in mice have permitted an extensive mechanistic dissection of how Aire operates. First, the availability of tissues and appropriate cell-sorting reagents permitted a clear delineation of where Aire is expressed. It is found primarily in lymphoid organs, especially the thymus — within the thymus it is expressed mostly by medullary epithelial cells (MECs) but also, although at much lower levels, by dendritic cells (DCs)[15,16]. Finding Aire in MECs was intriguing because of suggestions that this cell-type is involved in the negative selection of self-reactive thymocytes[17] and because of the coincident emergence of data establishing that transcripts encoding peripheral-tissue antigens (PTAs) are ectopically expressed specifically by MECs (reviewed in REF. 18). Thus, the hypothesis arose that Aire regulates the thymic transcription of genes encoding PTAs, and thereby controls thymocyte tolerization and consequently autoimmunity.

This hypothesis could be directly evaluated in *Aire*-knockout mice[15,19,20]. These animals were found to have a relatively normal immune system, but to be afflicted with multi-organ autoimmunity, as indicated by both inflammatory infiltrates and serum autoantibodies. Although the disease in *Aire*-knockout mice on a mixed C57BL/6 × 129/Sv genetic background was originally described to be relatively mild, a higher number of affected organs and a greater severity of disease were noted when the mutation was introduced into a variety of other mouse strains[21]. The autoimmune manifestations in *Aire*-knockout mice partitioned with Aire deficiency in thymic epithelial cells rather than in the differentiating thymocytes[15,22]. First, autoimmunity was observed in radiation bone-marrow chimaeras with Aire-deficient radio-resistant cells (including

Timeline | **The history of AIRE**



AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy.

epithelial cells) but not in chimaeras with Aire-deficient radio-sensitive cells (that is haematopoietic cells). Second, transfer of thymic epithelial cells from an *Aire*-knockout but not a wild-type mouse into a recipient athymic nude mouse led to autoimmunity.

Purification of MECs from *Aire*-knockout and wild-type mice followed by gene-expression profiling[15,16] revealed that Aire does indeed promote the transcription of a large number of PTA-encoding genes (such as those encoding insulin, salivary protein-1 and fatty-acid-binding protein). Interestingly, the transcription of a number of PTA genes, such as those encoding C-reactive protein and glutamic-acid decarboxylase of 67kDa (GAD67), appeared to be independent of Aire expression, and this observation was recently confirmed in a broader study[23]. Aire also positively or negatively regulates the transcription in MECs of a range of genes that do not encode PTAs. The significance of the control by Aire of PTA expression in the thymus for the autoimmune manifestations observed in *Aire*-knockout mice was recently substantiated by linking the loss of this control with the development of particular autoantibody specificities, specifically for the eye[24] and the stomach[25] and, in the former case, also demonstrating that the lack of PTA expression in the thymus was sufficient to produce autoimmunity.

It remained to be established how the control of PTA expression by Aire was translated into an effect on immunological tolerance. Through introduction of the *Aire*-knockout mutation into T-cell receptor (TCR)–self-antigen double-transgenic mice, in which self-antigen-specific T cells can be easily tracked, it was shown that, in the absence of Aire, self-reactive thymocytes can escape the usual clonal deletion that keeps them from emigrating to the periphery[16,22,26]. Although these studies clearly linked the control of PTA expression by Aire with clonal deletion, several reports have indicated that a lack of Aire compromised tolerance induction without affecting transcription of the corresponding PTA gene in the thymus[16,20,27]. Thus, there is now considerable interest in defining a role for Aire in the induction of central tolerance beyond its control of PTA expression. Evidence for a so-far-undefined effect on the presentation of antigens by MECs has been reported[16]. A schematic of the current view of how Aire expression in the thymus keeps autoimmunity in check is presented in FIG. 1.

AIRE and regulatory T cells

Aire clearly functions through the induction of T-cell tolerance in the thymus. But does it only partake in the 'recessive' mode of tolerance, by which ectopic PTA expression leads to clonal deletion of PTA-reactive thymocytes, or does it also elicit a 'dominant' mode of tolerance, by which the clonal deviation of PTA-reactive thymocytes into alternative lineages allows them to survive, while acquiring protective characteristics, for instance as forkhead box P3 (FOXP3)$^+$ T_{Reg} cells?

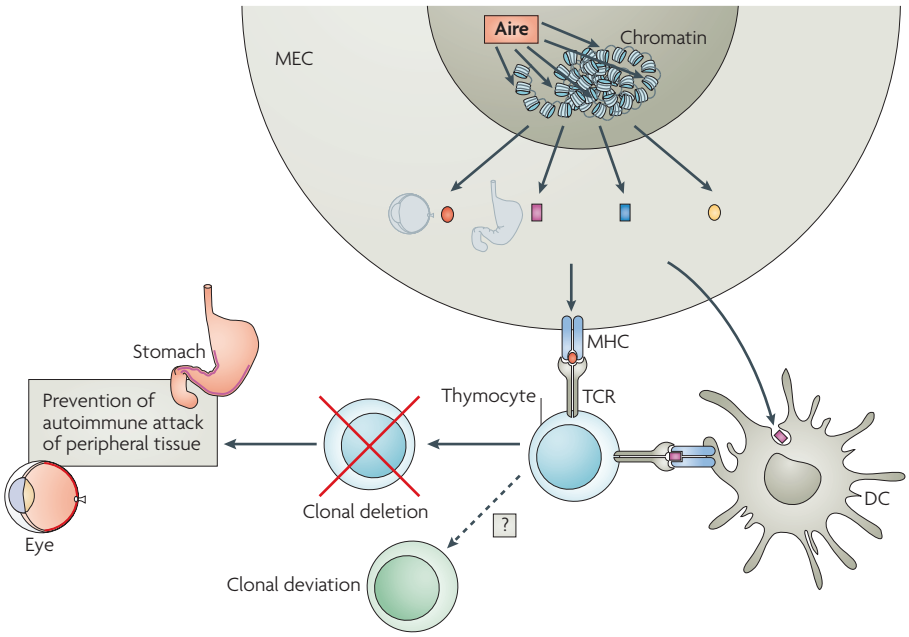


Figure 1 | **Aire: from transcriptional regulation to tolerance induction.** Autoimmune regulator (Aire) promotes the ectopic transcriptional activity of a large number of chromosomal locations, thereby enhancing the expression by medullary epithelial cells (MECs) of genes that would normally only be expressed in specific tissues. This 'shadow' of the peripheral self in MECs is then presented to immature thymocytes, either directly by the MECs themselves, or indirectly by uptake of antigens released from MECs by thymic dendritic cells (DCs). Differentiating T cells that recognize these antigens are then removed primarily by apoptotic clonal deletion, although some may survive by adopting alternative fates that have regulatory rather than autoreactive properties. These mechanisms thus prevent the autoimmune attack of peripheral organs. There is a clear match between the antigens that are ectopically expressed under the dictates of Aire and the specific antigenic targets that manifest in its absence. TCR, T-cell receptor.

Aire does not seem to grossly affect the differentiation of thymocytes towards the T_{Reg}-cell lineage on a global level. The numbers of $CD4^+CD25^{hi}FOXP3^+$ T_{Reg} cells in *Aire*-deficient mice are normal, and these cells show normal activity in the standard *in vitro* suppression assay[16,20,28]. By contrast, one report described a lower proportion of circulating $CD4^+FOXP3^+$ T cells in some patients with established APECED[28]. However, it is important to substantiate these data by analysing patients at an early stage of disease in order to distinguish cause from effect; that is, the observed reduction in circulating T_{Reg} cells might be secondary to the chronic fungal infection and autoimmune inflammation in these individuals, due to sequestration of T_{Reg} cells in the inflamed sites (as has been previously shown to occur in patients with HIV)[29]. Another argument against a global defect in T_{Reg} cells in AIRE-deficient individuals stems from the very different and aggressive nature of the autoimmune phenotypes in patients with immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) versus APECED, and in scurfy mice versus $Aire^{-/-}$ mice. In addition, there was a clear synergy in the effects of mutations in *Foxp3* and *Aire*: the disease in mice deficient in both factors was substantially worse than that in either single-mutant mouse[30], indicating that Aire has an impact on autoimmunity even in the absence of T_{Reg} cells.

Evidence from transfer experiments also argues against primary perturbations of a dominant mode of tolerance, as tested most directly in 'dual-thymus' transfer experiments, which entail the introduction of both a wild-type and an *Aire*-deficient thymus into athymic nude mice[16]. If the autoimmune disease imparted by the maturation of T cells in an Aire-deficient thymus is due to defective T_{Reg} cells, it should have been prevented by the generation of regulatory T cells, of any subset, by the co-grafted wild-type thymus; this was not the case.

The issue of the influence of Aire on T_{Reg} cells was also examined at the level of single self-reactive TCR specificities through crossing pairs of transgenes encoding a TCR and its cognate antigen into wild-type or *Aire*-knockout backgrounds. The results from several different double-transgenic systems of this nature showed that clonal deletion was affected by the absence of Aire[16,22,26], but that there was no significant effect on the number of $FOXP3^+$ T_{Reg} cells generated. Very recently, Aschenbrenner *et al.* directed the expression of an epitope from influenza haemagglutinin to $Aire^+$ thymic MECs, by relying on a transgene driven by the *Aire* promoter, and observed an increase in the generation of T_{Reg} cells of the corresponding specificity[31]. However, this study fell short of demonstrating that Aire has an important function in moulding the T_{Reg}-cell repertoire, as suggested by the authors, because it failed to establish that the ability of $Aire^+$ MECs to select T_{Reg} cells actually depends on Aire.

At this point, then, we would argue that there has not yet been a clear demonstration of an effect of Aire on the selection of T_{Reg} cells in the thymus, nor of a direct influence of Aire on the peripheral T_{Reg}-cell repertoire in a system that is free of confounding autoimmunity, immunodeficiency and infection. Nonetheless, because Aire does control the expression of PTAs in thymic MECs, it remains an attractive idea that it can drive clonal deviation of certain thymocytes towards the T_{Reg}-cell lineage rather than their clonal deletion, depending, for example, on the affinity and/or avidity of the TCR–MHC–PTA interaction.

Aire and dendritic cells

Aire is expressed in peripheral tissues, most notably in DCs, albeit at far lower levels than in thymic MECs[15,32,33]. It is found in both the $CD8^+$ and $CD8^-$ DC subsets, begging the question of its functional relevance, if any, in these cells. In addition, the root of the susceptibility of patients with APECED to *Candida albicans* infection (a disease hallmark) remains obscure. Several investigators have reported alterations in the antigen-presenting capabilities of myeloid cells derived from patients with APECED, albeit, paradoxically, as an enhanced effectiveness[34,35]. It will again be important to distinguish between cause and effect when assessing the significance of these divergences, but they may eventually explain the increased susceptibility of these patients to fungal infections.

On the other hand, the activity of AIRE in DCs is unlikely to really contribute to the induction of tolerance, either central or peripheral. Whereas *Aire* transcription is detectable by very sensitive PCR assays, the expression is very low in DCs compared with MECs — RNA transcripts are reduced by a factor of 10 or more as determined by quantitative RT-PCR analyses, and the protein is undetectable by flow cytometry using a sensitive intracellular staining technique that gives a clear signal in MECs[15] (D. Gray, C.B. and D.M., unpublished observations). In addition, the expression of Aire by DCs has essentially no effect on the transcription of PTA genes, the expression of which is strongly dependent on the expression of Aire in MECs[35] (E. Venanzi, D.M. and C.B., unpublished observations). Most directly, experiments on radiation bone-marrow chimaeras have shown that the autoimmune manifestations in $Aire^{-/-}$ mice develop independently of a defect in Aire expression in

Glossary

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
(APECED). A rare human autoimmune disorder that is inherited in an autosomal recessive manner and is characterized by various endocrine deficiencies, chronic mucocutaneous candidiasis and ectodermal dystrophies. It is caused by a number of different mutations in the gene that encodes autoimmune regulator (AIRE).

E3-ubiquitin ligase
An enzyme that is required to attach the molecular tag ubiquitin to proteins. Depending on the position and number of the ubiquitin molecules that are attached, the ubiquitin tag can target proteins for degradation in the proteasomal complex, sort them to specific subcellular compartments or modify their biological activity.

Non-obese diabetic mice
(NOD mice). Mice that spontaneously develop a form of autoimmunity that closely resembles human type 1 diabetes.

Nude mice
Mice homozygous for a mutation in the *Foxn1* gene, which causes both hairlessness and defective formation of the thymus, and therefore results in a lack of mature T cells.

PML nuclear bodies
One type of nuclear speckles of unknown function that contains several proteins, including the promyelocytic leukaemia protein PML.

Scurfy mice
Mice with a spontaneous mutation in the FOXP3 transcription factor (also known as Scurfin), which leads to a rapidly fatal lymphoproliferative disease, causing death by about 4 weeks of age. FOXP3-deficient mice lack the population of $CD25^+$ regulatory T cells.

SP100 family of transcriptional co-activators
The nuclear-matrix-associated protein SP100 belongs to a family of related proteins that contain nuclear-localization signals, dimerization domains and DNA-binding domains. They interact with other transcription factors to co-activate gene transcription.

Stromal cells
Cells of non-lymphoid origin that form the framework of each organ. These cells can support adhesion, proliferation and survival of distinct cell subsets.

Tolerance
A term that denotes lymphocyte non-responsiveness to antigen, but implies an active process, not simply a passive lack of response.

Box 1 | **The next decade of AIRE?**

The past 10 years have seen the identification of autoimmune regulator (AIRE) as a critical element in an important mechanism of immunological tolerance, and the delineation of some of the cellular and a few of the molecular mechanisms by which it operates. Some of the important questions to answer over the next decade include:

Molecular mechanisms

- Precisely how does AIRE control the ectopic expression of a range of genes that encode peripheral-tissue antigens (PTAs) and other proteins, the transcriptional regulation of which is widely divergent in their usual cellular locations?
- Does AIRE bind directly to DNA and/or to nucleosomes? If so, where does it bind?
- What other proteins does AIRE partner with?
- Do post-translational modifications control the activity of AIRE?
- How is the expression of *AIRE* controlled?
- What controls *AIRE*-independent PTA expression?

Developmental issues

- Does AIRE affect the differentiation of thymic medullary epithelial cells (MECs)? If so, how?
- Does AIRE influence the survival of MECs? If so, how?
- During what age-window is AIRE important?

Immunological issues

- What is(are) the additional role(s) of AIRE in the clonal deletion of thymocytes?
- Why are some peripheral organs attacked in the absence of AIRE and others not?
- What other cellular players of the innate or adaptive immune systems participate in the disease manifestations seen in AIRE-deficient individuals?
- Why do patients with APECED almost universally develop candidiasis infections?

Treatments?

- What would be an effective and the least invasive means of re-establishing tolerance in individuals who lack AIRE?

radio-sensitive myeloid cells, and instead are associated with a defect in Aire expression in radio-resistant stromal cells[15,22]. Thus, whereas Aire expression in peripheral DCs may affect the presentation of foreign antigens and susceptibility to fungal infections, it is of no evident consequence for the establishment and maintenance of tolerance to self.

It may also be worth mentioning that we do not currently know whether the situation is similar with regards to a potential role for Aire in lymph node stromal cells. It was recently reported that transcripts corresponding to Aire and certain PTAs were detectable at low levels in stromal cells of certain peripheral lymphoid organs; however, it has not yet been shown that the PTA transcripts depend on Aire expression, nor that Aire has any required function in these cells[36].

Molecular mechanisms

The molecular mechanisms underlying the function of Aire — that is, precisely how this protein promotes ectopic transcription of genes encoding PTAs and certain other proteins while repressing other loci — has been a topic of considerable interest. This issue is all the more intriguing because recent bioinformatic re-evaluations of the impact of Aire transcription have shown that it influences the expression of even more genes than previously thought, in the order of thousands rather than hundreds, representing a significant fraction of the total genome[23] (E. Venanzi, D.M. and C.B., unpublished observations).

Aire has many features of a transcription factor, sharing several domains with known transcriptional control elements: its SAND domain is homologous to the DNA-binding domains of members of the SP100 family of transcriptional co-activators[37,38]; it contains two PHD motifs, which are frequently found in nuclear transcriptional regulators[39]; and it has a nuclear localization signal. In MECs, AIRE is predominantly located in the nucleus, typically in punctate structures reminiscent of PML nuclear bodies[32,40]. AIRE can also modulate the transcription of reporter genes in co-transfection assays[41–43], and associates with other transcription factors, such as CREB-binding protein (CBP), at least *in vitro*[41,44].

On the other hand, several of the features of AIRE distinguish it from a 'conventional' transcription factor that binds to a well-defined DNA motif residing in the enhancer or promoter regions of a co-ordinately regulated set of genes. Indeed, the impact of AIRE on ectopic PTA gene expression is not that of a simple on/off operon[45]: its effect on most genes is only partial, and a number of PTAs are not affected by AIRE. In addition, the very breadth of its transcriptional impact is difficult to reconcile with the notion of a classical site-specific control element, as it is not easy to envision that an AIRE-binding site occurs in thousands of promoters of such disparate structure. Indeed, it has been difficult to obtain convincing evidence of DNA-binding specificity for AIRE, as existing data are questionable or internally inconsistent[46,47]. In addition, amino acids that are important for DNA binding by other SAND domains are altered in AIRE[9].

Recently, it was found that the start sites of ectopic PTA gene transcripts in MECs are often different (subtly or dramatically) from those for the same genes in the tissue where they are principally expressed (W. Besse, C.B. and D. M., unpublished observations). This finding argues against a model wherein AIRE would potentiate the transcription complexes that control these PTA genes in their usual cellular locations by enhancing either their expression or activity. Finally, the homology of AIRE with the SP100 proteins, and the association of AIRE with the nuclear matrix following transfection[40,43] may suggest an indirect mode of action, in which it would act by broadly modifying the nuclear sub-localization of chromosomal regions. The clustering of Aire-affected genes[48] is consistent with this view. Its effects on genes within the clusters tend to be heterogeneous, with activated or repressed genes interspersed with insensitive loci. Whereas these observations seem to rule out a simplistic explanation by which AIRE would open large chromatin loops to broadly activate transcription, more complex scenarios wherein long-range opening of a loop would permit activators or repressors to operate at short range remain possible.

The notion of AIRE as an epigenetic modifier of transcription is also consistent with a report that one of its PHD domains has E3-ubiquitin ligase activity[49], which might enable it to modify other nuclear factors by enhancing their operation or promoting their degradation. However, this ligase activity was questioned in a subsequent study[50].

Differences in AIRE in humans and mice?

Some investigators have speculated that in humans AIRE may serve a function different from or in addition to its role in mice. One of the major arguments for this view is that the autoimmune disorder first reported

in *Aire*-knockout mice was milder than the disease described in patients with APECED, and involved a different range of organs[15,19]. Also, it was found that Aire-deficient mice on the C57BL/6 genetic background have a profile of autoantibodies different from that of humans who have a defect in AIRE[51]. However, the observation that the identity and number of organs subject to autoimmune attack in mice devoid of *Aire* varied substantially according to the genetic background, and was very severe on certain backgrounds, such as in non-obese diabetic mice (NOD mice)[21], invalidated these arguments. It is quite expected that mice and humans would show a different spectrum of target organs: after all, this autoimmune disease results from an immune response, and, like any immune response, it is subject to MHC- or HLA-restriction, implying that not all individuals will be able to respond to the same autoantigens.

Another difference that has been reported between the two species is that some patients with established APECED, but not *Aire*-knockout mice on the C57BL/6 or a mixed C57BL/6 × 129/Sv genetic background, have a defect in the representation and function of T_{Reg} cells[28]. But, as mentioned above, it will be important to substantiate this difference in the absence of the confounding *C. albicans* infection in patients and at comparable levels of background autoimmunity. The *C. albicans* infection itself represents an interesting, and striking, divergence between patients with APECED and mice lacking *Aire*, but we do not yet know whether the dissimilarity is a reflection of AIRE operating in a different manner in the two species or whether it relates more to differences in physiology (for example, of the skin) or in the environment. In this regard, the recent observation of autoantibodies specific for interferon-α (IFNα) and IFNω in most patients with APECED, but not in *Aire*-knockout mice, is intriguing[52]. We would like to suggest that, whereas it is important to be aware of species-dependent disparities in AIRE function, to date no convincing evidence of this exists.

Perspectives

The cloning of *AIRE* as the principal gene responsible for APECED was a leap forward, particularly in our comprehension of this devastating autoimmune disease and, more generally, in our understanding of how autoimmune disorders can arise from a defect in T-cell tolerance. For example, the role of polymorphisms in the number of variable nucleotide repeats (VNTRs) in the promoter region of the human gene encoding insulin in promoting susceptibility to type 1 diabetes[53,54] suggests that similar central tolerance mechanisms may be at play in autoimmune diseases other than APECED. The identification of the role of Aire in controlling the ectopic expression of PTAs in thymic MECs in mice, and the establishment of thymocyte clonal deletion as the primary cellular mechanism for determining the impact of Aire have been important elements in a general re-awakening of interest in central tolerance, in the wake of an emphasis on peripheral tolerance occasioned by the reigning wave of enthusiasm over T_{Reg} cells. As outlined in BOX 1, many questions about AIRE and APECED (and their mouse counterparts) remain to fascinate us for the next decade.

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Competing interests statement

The authors declare no competing financial interests.

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