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EXPERT OPINION

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Thymosin α 1: burying secrets in the thymus

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Thymosin α 1 (T α 1), an epithelial cell (EC)-derived cytokine, has the strong ability to modulate signals delivered through innate immune receptors on dendritic cells (DCs), thus instructing the initiation of appropriate immune responses to T cells. In its ability to activate indoleamine 2,3-dioxygenase 1-dependent tolerogenic programs in DCs, T α 1 pivotally contributes to the maintenance of self-tolerance by regulating the function of regulatory T (Treg) cells. How T α 1 may contribute to the Treg cell ontogeny is not known. The transcriptional regulator autoimmune regulator (AIRE) is known to control central and peripheral tolerance. AIRE is highly expressed in thymic medullary ECs where it controls the ectopic expression of tissue restricted antigens for negative selection. The absence of AIRE-induced tissue-specific antigens in the thymus can lead to autoimmunity in the antigen-expressing target organ. Recently, AIRE protein has been detected in peripheral lymphoid organs, suggesting that peripheral AIRE may play a complementary role. We have addressed the possible relationship between AIRE and T α 1 and discovered an intricate crosstalk, whereby AIRE may promote prothymosin cleavage to T α 1, and T α 1 in turn transcriptionally regulates AIRE expression. Thus, similar to other members of thymic stromal poietins, T α 1 expressed within the thymus and peripheral tissues regulates the EC/DC crosstalk required for salutary immune homeostasis.

Keywords: autoimmune regulator, indoleamine 2,3-dioxygenase, immune tolerance, thymosin α 1

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1. Introduction

Thymosin α 1 (T α 1, commercial name ZADAXIN[®]) is a naturally occurring thymic peptide first described and characterized by Goldstein and Goldstein and Garaci [1,2]. T α 1 is found in highest concentrations in the thymus. However, it is produced in small amounts in several peripheral lymphoid and non-lymphoid, tissues, such that circulating levels measured in healthy adults by immunoassays are in the 252 – 1158 pg/ml range [3]. Prepared as a 28mer synthetic amino-terminal acylated peptide, T α 1 is in clinical trials worldwide for the treatment of some viral infections, either as a monotherapy or in combination with IFN- α [4]. Additional indications are some immunodeficiencies, malignancies and HIV/AIDS [5]. T α 1 has shown an excellent safety profile and does not induce the side effects and toxicities commonly associated with immunomodulatory agents. The mechanism of action of the synthetic polypeptide is thought to be related to its immunomodulating activities, centered primarily on the augmentation of T-cell function. To do this, T α 1 primarily acts on cells of the innate immune system, including macrophages, neutrophils and dendritic cells (DCs) [6-8]. As such, T α 1 acts as an endogenous regulator of both the inflammatory and adaptive immune responses [6,9].

2. From pleiotropy to molecular mechanisms of a system-oriented molecule

The complex pathophysiology of inflammatory-driven immune and autoimmune diseases, from infections to cancer, is self-amplifying and redundant at multiple levels. Thus, the intrinsic robustness of living systems against various perturbations is a key factor that prevents most anti-inflammatory drug from being fully successful. This implicates that pharmacological therapy for inflammatory diseases would only be successful if addressing these complex network systems. Any successful drug would have to be pleiotropic, and work on the many components of the inflammatory/autoimmune cascade at a time. T α 1 exceptionally fulfils this requirement, qualifying itself as a context-dependent molecule capable of multi-target interactions in physiological and non-physiological conditions. Despite living up to its promises, T α 1 continues to amaze us with its pleiotropy, multi-targeting activity, and ability to utilize different molecular mechanisms depending on context. Recent insights into the molecular mechanisms of T α 1's pleiotropy have revealed its central role in modulating DC function, which is consistent with the ability of T α 1 to induce different forms of immunity and tolerance [10]. Results have shown that T α 1: i) primed DCs for antifungal T-helper (Th)1 resistance through Toll-like receptor (TLR)/MyD88-dependent signaling and this translated *in vivo* to protection against fungal infections [6]; ii) activated plasmacytoid DCs via the TLR9/MyD88-dependent viral recognition, thus leading to the activation of IFN regulatory factor 7 and the promotion of the IFN- α /IFN- γ -dependent effector pathway, and this resulted in protection against primary murine cytomegalovirus infection [11]; and iii) induced indoleamine 2,3-dioxygenase (IDO)1 activity in DCs, thus affecting tolerization toward self as well as microbial non-self antigens, and this resulted *in vivo* in transplantation tolerance [8] and protection from inflammation and allergy [12]. Evidence suggests that the ability of T α 1 to signal through disparate TLRs on DC subsets may occur through the interaction with multiple, yet distinct, TLR ligands, either endogenous or exogenous [10]. Heterocomplexes and collaborative interactions between endogenous and/or exogenous TLR agonists and T α 1 may lead to novel reciprocal trends of immune surveillance and effector mechanisms aiding safe disposal of pathogens and/or pathogenic insults whereby excessive inflammatory responses and tissue injury may effectively be prevented. NF- κ B, a family of seven structurally related transcription factors that play a central role in the stress response and inflammation [13], are exploited by T α 1 for its pleiotropic activity [10]. This finding not only qualifies T α 1 as a context-oriented molecule but may also offer molecular explanations for a successful drug working on the many components of the inflammatory/immune response, that is, for successful pleiotropy.

3. Promoting tolerance by T α 1 via tryptophan catabolism

The induction of tolerance is critical for the maintenance of immune homeostasis. Within the new paradigm of resistance and tolerance mechanisms as two alternative but complementary host defense strategies [14], boosting tissue tolerance is likely to be a useful strategy in infectious and non-infectious diseases. The mammalian intestinal and respiratory mucosal surfaces are continuously exposed to a complex and dynamic community of microorganisms. These microbes establish symbiotic relationships with their hosts, making important contributions to immune fitness and metabolism efficiency [15,16]. The immune system is pivotal in mediating the interactions between host and microbiota that shape the mucosal environment. At the mucosal surfaces, a system of tolerance and controlled inflammation to limit the response to dietary or pathogen-derived antigens is present. This regulation is achieved by a number of cell populations acting through a set of shared regulatory pathways [17,18]. Regulatory T (Treg) cells constitute a pivotal mechanism of immunological tolerance. The potential role of malfunctioning Treg cells in chronic inflammatory immune and autoimmune diseases is well-documented [19]. Recent studies have demonstrated a crucial role for tryptophan catabolism and kynurenine production in the induction of peripheral tolerance [20,21]. IDO1 is an intracellular heme-containing enzyme that catalyzes the initial and rate-limiting step in tryptophan degradation along the kynurenine pathway. IDO1 is widely expressed in a variety of human tissues as well as in macrophages and DCs and is induced in inflammatory states by IFN- γ and other proinflammatory cytokines. Studies have demonstrated a complex and crucial role for IDO1 in immunoregulation during infection, pregnancy, autoimmunity, transplantation and neoplasia [22]. By inducing IDO1 and promoting Treg cells, T α 1 successfully ameliorated respiratory allergy and intestinal inflammation in different experimental models [12]. Thus, maintaining diplomatic relations between mammals and microbial communities could be added to the therapeutic and immunomodulatory properties of T α 1.

4. En route to tolerance: T α 1 meets AIRE

The transcription factor autoimmune regulator (AIRE) is encoded by the homonymous gene, *AIRE*, situated in the region q22.3 of chromosome 21 [23,24]. The homologous murine gene, *AIRE* is located in the chromosome 10 and shows 77% nucleotide correspondence with human *AIRE* [25]. AIRE expression displays the highest levels in the thymus, especially in medullary epithelial cells (mTECs) and, less markedly, in DCs. Peripheral lymph nodes, fetal liver and spleen also express AIRE, albeit at low levels [26]. Consistent with its high rate of expression in mTECs, AIRE is considered

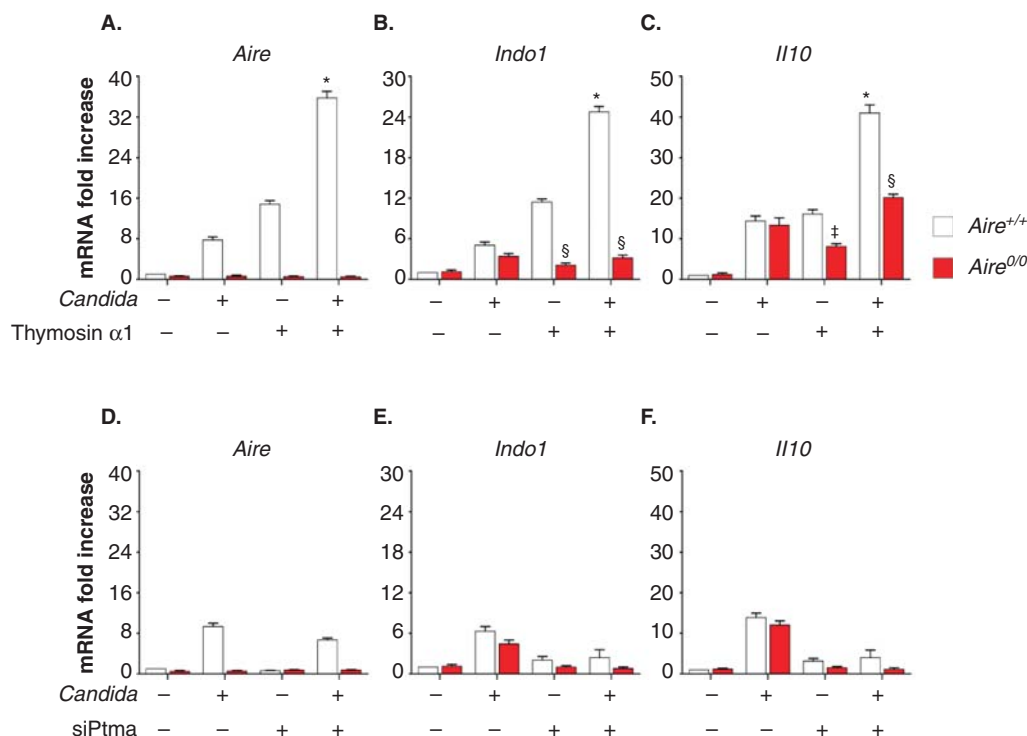


Figure 1. T α 1 promotes *Aire* and *Indo1* gene expression on murine ECs. ECs were exposed to *Candida albicans* and assessed for *Aire* (A and D), *Indo1* (B and E) and *Il10* (C and F) gene expression in the presence of T α 1 (A – C) or siPtma (prothymosin alpha) (D – F). ECs were purified from murine intestine as described [40] and stimulated (10^6) with inactivated *Candida* yeasts (1:1 ratio), with and without 100 μ g/ml of T α 1 (SciClone Pharmaceuticals, Inc) for 12 h at 37°C. ECs were exposed to 1 μ M siPtma (Dharmacon RNAi Technologies) for 72 h, as described [42] before the addition of *Candida*. Gene expression was evaluated by real-time RT-PCR (Stratagene) with specific primers [42]. The *Aire* primers were as follows: Forward, 5'-CCTGGATTTCTGGAGGATTCT-3'; reverse, 5'-CCGTCCAGGATGCTATGCA-3'. * $p < 0.05$, $^{\dagger}p < 0.01$, $^{\S}p < 0.001$, T α 1-treated versus untreated cells and *Aire*^{0/0} versus *Aire*^{+/+} cells. The data are pooled from two experiments.

Aire: Autoimmune regulator; EC: Epithelial cells; *Indo1*: Indoleamine 2,3-dioxygenase; RNAi: RNA interference; RT-PCR: Real-time polymerase chain reaction; T α 1: Thymosin α 1.

a major player towards the acquisition of central tolerance. The mTECs express a large number of genes, including tissue-specific antigens (TSAs) that are normally present only in specialized peripheral organs and are apparently not required for the direct functioning of mTECs [27]. During negative selection, these encoded TSAs are presented by mTECs or DCs as self-antigens to differentiating thymocytes, leading to the induction of tolerance either by clonal deletion, functional inactivation of self-reactive T cells or clonal deviation [28,29]. The thymic expression of many of these TSAs is dependent on the *AIRE* gene [30] and mutations in *AIRE* lead to severe, multiorgan, tissue-specific autoimmunity in both mice and humans [28]. Although these results reveal a role of thymic *AIRE*, self-tolerance must continue to be enforced after T cells leave the thymus. In this regard, human peripheral lymphoid tissues contain *AIRE*-expressing DCs [31] and a population of extrathymic *AIRE*-expressing cells (eTACs) that may play an important and previously uncharacterized role in self-tolerance have been described [32]. The eTACs share certain characteristics with mTECs, including

being equipped to act as professional antigen-presenting cells and the *AIRE*-regulated expression of TSAs. The set of *AIRE*-regulated TSAs expressed in eTACs appears to have little overlap with thymic *AIRE*-regulated antigens. The lack of overlap suggests that there may be a higher order of *AIRE*-dependent transcriptional regulation of TSA expression, whether direct or indirect, that differs between the thymus and the periphery. The eTACs may represent a safety net within the entire immunological periphery, which functions to screen out naïve autoreactive T-cell clones that escape thymic negative selection and, finally, may play an increasingly important role with advancing age, as the thymus involutes and the burden of maintaining self-tolerance shifts to the periphery [32].

With *AIRE* influencing intrathymic and extrathymic expression of numerous TSAs [28], it becomes apparent that the regulation of *AIRE* will be an important determinant in self-tolerance control. T α 1 is highly expressed in human thymus (at the levels ranging from 45.0 to 52.1 ng/mg protein in normal thymuses) [33] and is localized on ECs, lying

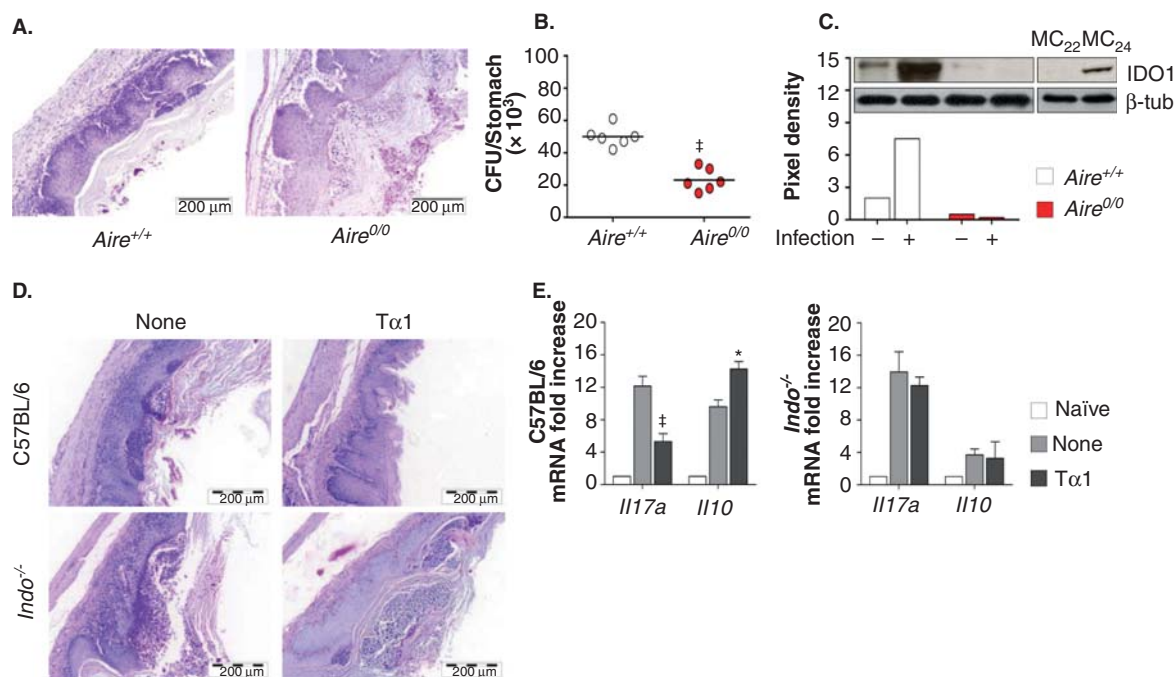


Figure 2. Aire controls IDO1 protein expression in vivo. *Aire*^{0/0} and *Aire*^{+/+} mice on the BALB/c background were injected intragastrically with 10⁸ *Candida albicans* and assessed a week later for (A) stomach histology by periodic acid-Schiff (PAS) staining (note the presence of abundant inflammatory cells and signs of acanthosis and parakeratosis in the stomachs of *Aire*^{0/0}) and fungal growth (B), expressed as CFU per organ (mean ± SE). (C) IDO1 expression in mesenteric lymph nodes (immunoblotting was performed with rabbit polyclonal IDO1-specific antibody, as described in Ref. [43]. The positive control consisted of IDO1-expressing MC₂₄ transfectants and the negative control of mock-transfected MC₂₂ cells). Corresponding pixel density ratio was normalized against β-tubulin. ***p = 0.001, *Aire*^{0/0} versus *Aire*^{+/+} mice. The data reported are from one experiment representative of three with similar results. (D) Stomach histology (PAS staining) is shown and (E) *Il17a* and *Il10* expression shown on stomach total cells from C57BL/6 or *Indo*^{-/-} mice intragastrically infected with 10⁸ *C. albicans*, treated with 200 μg/kg/i.p. of Tα1 daily for a week and immediately sacrificed. Images were acquired with a 40× objective. Bars indicate magnification. Data are representative (histology) or pooled from two experiments. *p < 0.01, ‡p < 0.001, Tα1-treated versus untreated (none) mice (naïve, uninfected and untreated mice).

Aire: Autoimmune regulator; IDO1: Indoleamine 2,3-dioxygenase; Tα1: Thymosin α1.

singly, grouped, in Hassall's corpuscles [34]. Human Hassall's corpuscles also express thymic stromal lymphopoietin (TSLP) that is known to condition thymic CD11c-positive DCs to induce the proliferation and differentiation of CD4⁺CD8⁻CD25⁻ thymic T cells into CD4⁺CD25⁺ forkhead box P3 (FoxP3⁺) Treg cells [35]. These findings suggested that Hassall's corpuscles have a critical role in DC-mediated secondary positive selection of medium-to-high affinity self-reactive T cells, leading to the generation of CD4⁺CD25⁺ Treg cells within the thymus. It is of interest that human TSLP appears to induce expression of AIRE in human DCs [36], although the functional significance and *in vivo* relevance of this finding is not known.

5. Tα1 promotes AIRE expression

Given the many functional similarities between TSLP [37] and Tα1 [12], we hypothesized that Tα1 could also be able to activate AIRE expression on ECs. To this purpose, we have

assessed the ability of Tα1 to activate AIRE expression on murine ECs isolated from the gastrointestinal tract of naïve mice and exposed to the fungus *Candida albicans in vitro*. We found that Tα1 significantly increased AIRE expression on ECs (Figure 1A). On the same cells, Tα1 also induced the expression of *Indo1* gene coding for IDO1 (Figure 1B) and *Il10* gene coding for IL-10 (Figure 1C) which were both dependent on AIRE, being negated, at least partially, in *AIRE*^{0/0} ECs (Figure 1A – C). To determine whether endogenously produced Tα1 also regulates the expression of these genes, we assessed AIRE, *Indo1* and *Il10* gene expression on ECs on inhibition of prothymosin alpha (*Ptma*) by specific small interfering RNA (siRNA). It is known that Tα1 is produced *in vivo* by cleavage of *Ptma* in diverse mammalian tissues [38]. We found that AIRE, and even more, the *Indo1* and *Il10* genes failed to upregulate in response to the fungus in the presence of siRNA targeting the *Ptma* gene (Figure 1D – F). Preliminary as they are, these data suggest

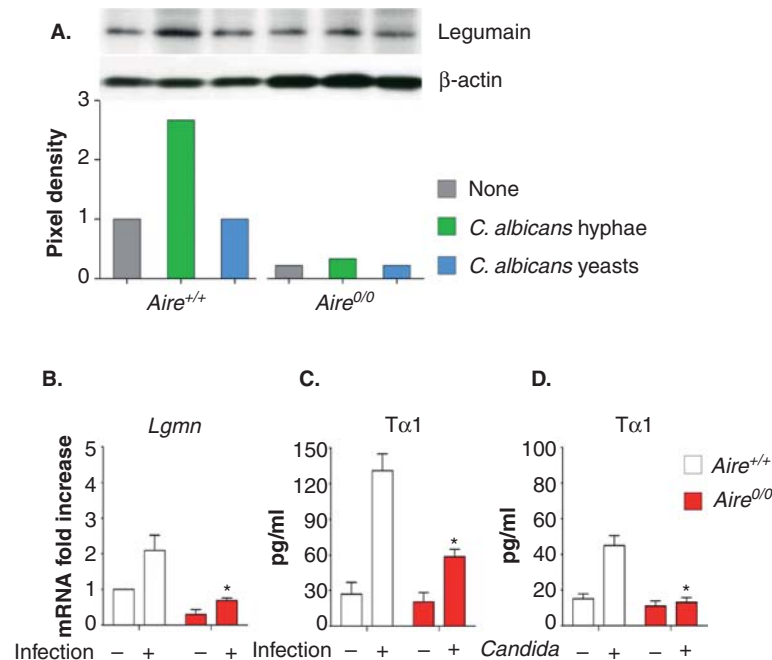


Figure 3. Aire promotes T α 1 production. (A) Legumain production in DCs purified from the mesenteric lymph nodes and pulsed for 6 h with live *Candida albicans* yeasts and hyphae (at the cell:fungi ratio of 1:1) as described [43]. Immunoblotting was done on cell lysates with the anti-legumain specific antibody (Santa Cruz). Corresponding pixel density ratio was normalized against β -actin. (B) *Lgmn* (Legumain) gene expression by RT-PCR and (C) T α 1 production (by specific ELISA from Immundiagnostik) in *Aire*^{0/0} and *Aire*^{+/+} mice a week after intragastric infection with 10^8 *C. albicans* are shown. Assays were done on stomach total cells or homogenates (pooled from 6 mice). (D) T α 1 production by intestinal ECs is isolated from naïve mice and stimulated with *Candida* as in legend to Figure 1). * $p < 0.01$, *Aire*^{0/0} versus *Aire*^{+/+} mice and cells. The data are pooled from two experiments.

Aire: Autoimmune regulator; DC: Dendritic cell; RT-PCR: Real-time polymerase chain reaction; T α 1: Thymosin α 1.

that T α 1 may contribute to *AIRE* expression and function that includes IDO1 activation and IL-10 production.

6. AIRE drives IDO1 production

We have already shown that *AIRE*^{0/0} mice on BALB/c background are much more susceptible than *AIRE*^{+/+} mice to mucosal candidiasis, a hallmark of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy disease caused by *AIRE* mutations [23,24]. IL-10⁺FoxP3⁺ T cells were significantly decreased in these mice, a finding pointing to a tolerance defect correlated with *AIRE* deficiency [39]. To assess whether, similar to the *in vitro* findings, AIRE regulates IDO1 expression *in vivo*, we assessed IDO1 protein expression in the mesenteric lymph nodes of *AIRE*^{0/0} and *AIRE*^{+/+} mice intragastrically infected with *C. albicans*. Consistent with previous data [39], *AIRE*^{0/0} mice were clearly more susceptible than *AIRE*^{+/+} to candidiasis, as revealed by the extensive inflammatory cell recruitment and signs of immunopathology in the stomach of *AIRE*^{0/0} mice (Figure 2A), likely accounting for the ability of these mice to better control the growth of the fungus as compared to *AIRE*^{+/+} mice (Figure 2B). In terms of IDO1 protein expression, we found that this expression was defective in

AIRE^{0/0} mice on infection (Figure 2C), a finding further pointing to the contribution of AIRE to the generation of self-tolerance [28]. These findings clearly suggest that T α 1 and AIRE cooperate in the induction of IDO1-dependent immune tolerance. As a matter of fact, lack of tolerance occurred in *Indo*^{-/-} mice infected intragastrically with *C. albicans*, as judged by signs of extensive immunopathology in the stomach (Figure 2D) and imbalanced *Il17a/Il10* gene expression (Figure 2E), as already demonstrated [40]. Of interest, treatment with T α 1 failed to restore immune tolerance in *Indo*^{-/-} mice (Figure 2D, E), a finding further pointing to IDO1 as an important mediator of T α 1-induced tolerance.

7. AIRE promotes T α 1 production

T α 1 is produced *in vivo* by cleavage of Ptma in diverse mammalian tissues [38]. Evidence indicates that a lysosomal asparaginyl endopeptidase, called legumain, is able to process Ptma to generate T α 1 [38]. *AIRE* expression in human monocyte-derived DCs was found to correlate with a slight downregulation of legumain-specific transcript in unstimulated cells [41]. We assessed legumain production in purified intestinal DCs exposed to *C. albicans* yeasts or hyphae. Surprisingly, we

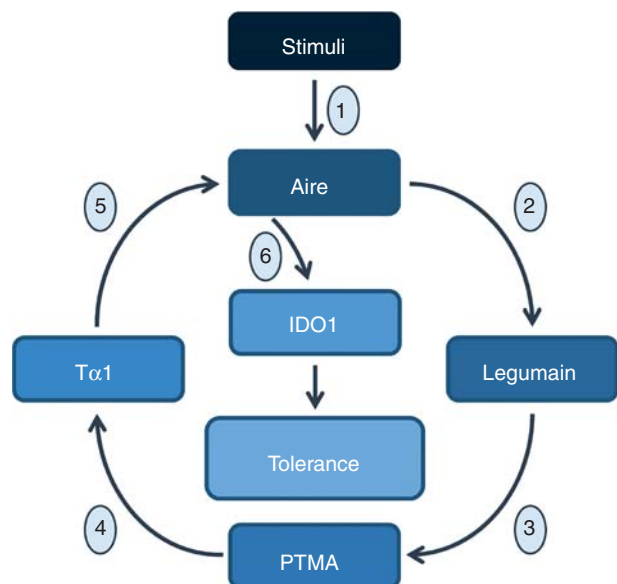


Figure 4. Schematic representation of the possible pathways underlying the Tα1/Aire synergistic crosstalk in tolerance. Induced in infection (1), Aire may activate the asparaginyl endopeptidase legumain activity (2) to PTMA cleavage (3) leading to the production of Tα1 (4) that in turn activates Aire transcription (5) which is required for the activation of the enzyme IDO1 (6).

Aire: Autoimmune regulator; IDO1: Indoleamine 2,3-dioxygenase 1; PTMA: Prothymosin α; Tα1: Thymosin α1.

found a significant increase in the production of legumain in DCs exposed to the hyphae, an effect that was not observed in *AIRE*^{0/0} DCs (Figure 3A). Therefore, *AIRE* expression correlates with upregulation of legumain protein production. To assess whether a similar regulation occurs *in vivo*, we evaluated *legumain* expression in the stomach of *AIRE*^{0/0} and *AIRE*^{+/+} mice intragastrically infected with *C. albicans*. We found a profound downregulation of *legumain* mRNA expression in conditions of *AIRE* deficiency (Figure 3B), associated with a

lower Tα1 production (Figure 3C). Lower Tα1 production was similarly observed in purified *AIRE*^{0/0} ECs on *in vitro* stimulation with *C. albicans* (Figure 3D), a finding suggesting that Aire may promote cleavage of Ptma in ECs via legumain.

8. Conclusion

The discovery of AIRE as an unexpected partner in Tα1's physiological role may represent an important advance in the field of therapeutic development of Tα1. Our data would indicate the existence of a crosstalk between AIRE and Tα1, whereby AIRE may promote Ptma cleavage to Tα1 through regulation of the asparaginyl endopeptidase legumain activity and Tα1, in turn, regulates AIRE expression at the transcriptional level (Figure 4). Although more work is needed to provide mechanistic insights governing the reciprocal regulation between AIRE and Tα1 in ECs and/or other types of cells and the possible *in vivo* significance, AIRE exploitation by Tα1 qualifies Tα1 as a master regulator of central and peripheral immune tolerance.

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Declaration of interest

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