THYMIC STROMAL ALTERATIONS AND GENETIC DISORDERS OF IMMUNE SYSTEM

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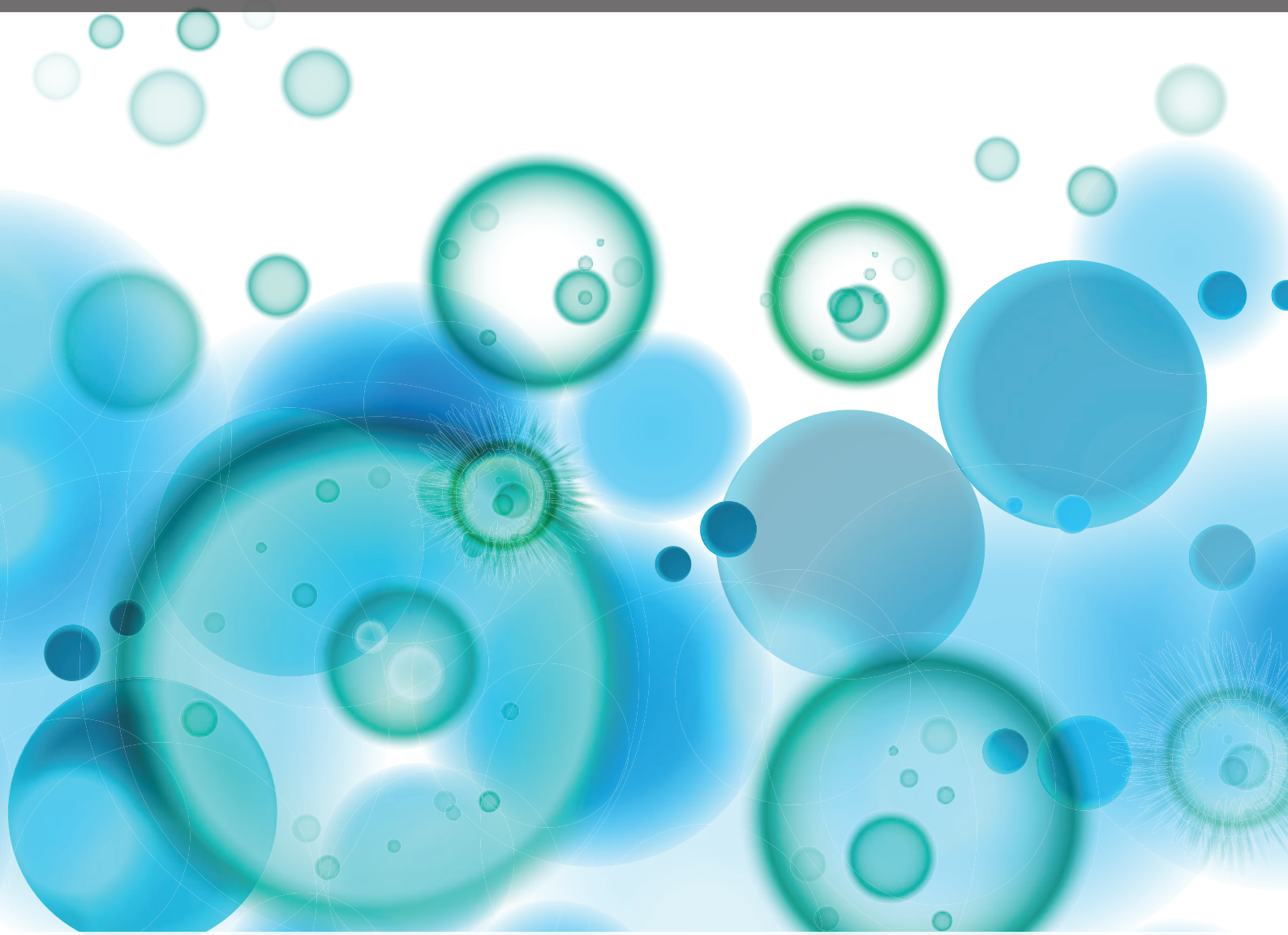

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THYMIC STROMAL ALTERATIONS AND GENETIC DISORDERS OF IMMUNE SYSTEM

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The pathogenic mechanisms underlying primary T-cell disorders are mainly related to molecular alterations of genes whose expression is intrinsic to hematopoietic cells. However, since the differentiation process requires a crosstalk among thymocytes and the thymic microenvironment, molecular alterations of genes, involved in the differentiation and functionality of the stromal component of the thymus, may lead to a severe T-cell defect or failure of central tolerance, as well. The first example of severe combined immunodeficiency (SCID) not related to an intrinsic alteration of the hematopoietic cell but rather of the thymic epithelial component is the Nude/SCID phenotype, inherited as an autosomal recessive disorder, whose hallmarks are the T-cell defect and the absence of the thymus. The clinical and immunological phenotype is the human equivalent of the murine Nude/SCID syndrome, which represents the first spontaneous SCID identified in nude mice in 1966. For over 3 decades studies of immune system in these mice enormously contributed to the overall knowledge of cell mediated immunity, in the assumption that the athymia of these mice was solely responsible for the T-cell immunological defect. This syndrome is due to mutations of the transcription factor FOXN1, belonging to the forkhead-box gene family, which is mainly expressed in the thymus and skin epithelial cells, where it plays a critical role in differentiation and survival. An alteration of the thymic structure is also a feature of the DiGeorge syndrome (DGS), which has been long considered the human counterpart of the nude mice phenotype. This syndrome is frequently associated to a deletion of the 22q11 region, which contains approximately 30 genes, including the TBX1 gene, which is responsible for most of the clinical features of DGS in humans and mice. In this syndrome common manifestations are cardiac malformations, speech delay, hypoparathyrodism and immunodeficiency, even though the immunological hallmarks of the T-cell defect in DiGeorge syndrome are profoundly different from those reported in human Nude/SCID. The divergence of the phenotype among these 2 entities raised the possibility that the FOXN1 transcription factor represents the real key stromal molecule implicated in directing the hematopoietic stem cell toward a proper T-cell fate. Thymic stromal component of the primary lymphoid organ is also required to negatively select the autoreactive clones, a process driven by the expression of tissue specific antigens (TSA) by medullary thymic epithelial cells (mTECs). The expression of genes encoding TSA antigens is mediated by autoimmune regulator (AIRE) gene, encoding a transcription factor expressed in

mTECs. Molecular alterations of this gene are associated to autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), a rare autosomal disorder, which may be considered the prototype of an autoimmune disease due to the failure of central tolerance homeostasis.

All these "experiments of nature" led to unravel novel pathogenic mechanisms underlying inherited disorders of immune system and, of note, to clarify the pivotal role of epithelial cells in the maturation and education process of T-cell precursors.

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EDITORIAL
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Thymic stromal alterations and genetic disorders of immune system

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Keywords: severe combined immunodeficiency, FOXN1, central tolerance, medullary thymic epithelial cells, DiGeorge syndrome, rag defects, APECED

In this specialty section of the journal, we host a topic focused on thymic stromal alterations and genetic disorders of immune system. The thymus is a specialized organ of the immune system where, through stage-specific differentiation of hematopoietic progenitor cells, fully mature and self-tolerant T cells origin. The process is strictly dependent on the link between the thymic stromal cells (TSCs), which allow the selection of a functional and self-tolerant T-cell repertoire, and the thymus tridimensional architecture. Indeed, the interaction between the developing thymocytes and the stromal cells is crucial for the development of both T cells and TSCs (1, 2). In both human and mice, the primordial thymic epithelial cells (TECs) are yet unable to fully support the T-cell development and only after the transcriptional activation of the *Forkhead-box n1* (*FOXN1*) gene, this essential function is acquired. Most of the information concerning the T-cell development came out from studies on mice carrying null mutation in *FOXN1* gene. In humans, as detailed in the Romano et al. review, the Nude/SCID phenotype is characterized by congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T-cell immunodeficiency, inherited as an autosomal recessive disorder (3). As extensively approached in the Villa et al. review, the intercellular cross-talk is also essential to support the maturation of Foxp3C natural regulatory T cells. In Omenn syndrome (OS), caused by hypomorphic Rag alterations, an infiltration of peripheral tissues by activated T cells and immune dysregulation have been found (4). The authors discuss on abnormalities of thymic microenvironment in OS with a special focus on the defective maturation of TECs, and impairment of central tolerance.

The commonest association of thymic stromal deficiency resulting in T-cell immunodeficiency is the DiGeorge syndrome (DGS), discussed in the Davies review. In this syndrome, however, the immunological impairment is highly variable, ranging from normal to a severe immune defect in rare individuals, thus suggesting that partial thymic hypoplasia may occur or that extrathymic sites of differentiation play a role in the process (5). The difference in the immunological defects between DGS and the Nude/SCID phenotypes implies that FOXN1 controlled genes are mandatory for a fully mature T-cell development process rather than the integrity of the thymus itself.

It is known that autoimmune regulator (*AIRE*) gene plays a central role in the induction of central tolerance, and different mechanisms of action have been hypothesized for this process. According to the most reliable theory, AIRE directly induces the production of tissue-specific antigens (TSA) (6). However, recent evidence suggests that another mechanism for negative selection of self-reactive thymocytes may be due to AIRE-induced differentiation of medullary TECs, and regulation of the expression of intrathymic chemokines directed to antigens presenting cells (APCs), such as thymocytes and dendritic cells (7). In their reviews, Laan and Peterson and Kisand et al. give an overview on what is known about the different mechanisms through which AIRE induces central tolerance.

The process aimed at the elimination of potential self-reactive T cells in the thymus is crucial for preventing the onset of autoimmune diseases. As discussed in the Akiyama et al. paper, medullary epithelial cells play a central role in the process through the regulation of gene expression, and, in particular, of those genes encoding for the TNF family cytokines, RANK ligand, CD40 ligand, and lymphotoxin. These genes promote the differentiation of AIRE- and TSA-expressing mTECs (8).

The mechanism by which a single *AIRE* gene can influence the transcription of such a large number of TSA within mTECs has been discussed in the Matsumoto et al. paper. Two models have been proposed. The first one implies a direct transcriptional control of *AIRE* on TSA, while the second one is based on the role of *AIRE* on the maturation program of mTECs (9).

The clinical and immunological phenotype of patients affected with autoimmune polyendocrinopathy ectodermal dystrophy (APECED), as reviewed by Petteri Arstila and Jarva, is characterized by multiple endocrine deficiencies, the most common manifestations being hypoparathyroidism, Addison's disease, hypogonadism, and secondary amenorrhea, usually associated with the presence of autoantibodies toward the target tissues (10). However, the phenotype and, therefore, the underlying pathogenic mechanism, are even more complex, in that Chronic Mucocutaneous Candidiasis is also a prominent part of the disease. This clinical entity is related to abnormalities in the Th17-related cytokines, which are mostly involved in immune defenses against Candida (11). Finally, high titers of neutralizing autoantibodies against type I interferons, which have been shown to downregulate the expression of interferon-controlled genes, have been documented (12).

In this Research Topic, De Martino et al. focus their attention on the complexity of the APECED phenotype in that a wide variability of the clinical expression, in the presence of the same genotype alteration, has been found (13). They suggest that additional

mechanisms, in addition to AIRE function, are involved in the pathogenesis of the disease. This might be helpful to understand not only the molecular basis of APECED but will also help improve diagnosis, management, and therapeutic strategies to treat this complex disease.

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FOXN1: a master regulator gene of thymic epithelial development program

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T cell ontogeny is a sophisticated process, which takes place within the thymus through a series of well-defined discrete stages. The process requires a proper lympho-stromal interaction. In particular, cortical and medullary thymic epithelial cells (cTECs, mTECs) drive T cell differentiation, education, and selection processes, while the thymocyte-dependent signals allow thymic epithelial cells (TECs) to maturate and provide an appropriate thymic microenvironment. Alterations in genes implicated in thymus organogenesis, including *Tbx1*, *Pax1*, *Pax3*, *Pax9*, *Hoxa3*, *Eya1*, and *Six1*, affect this well-orchestrated process, leading to disruption of thymic architecture. Of note, in both human and mice, the primordial TECs are yet unable to fully support T cell development and only after the transcriptional activation of the *Forkhead-box n1* (*FOXN1*) gene in the thymic epithelium this essential function is acquired. *FOXN1* is a master regulator in the TEC lineage specification in that it downstream promotes transcription of genes, which, in turn, regulate TECs differentiation. In particular, *FOXN1* mainly regulates TEC patterning in the fetal stage and TEC homeostasis in the post-natal thymus. An inborn null mutation in *FOXN1* leads to Nude/severe combined immunodeficiency (SCID) phenotype in mouse, rat, and humans. In *Foxn1*$^{-/-}$ nude animals, initial formation of the primordial organ is arrested and the primordium is not colonized by hematopoietic precursors, causing a severe primary T cell immunodeficiency. In humans, the Nude/SCID phenotype is characterized by congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T cell immunodeficiency, inherited as an autosomal recessive disorder. Aim of this review is to summarize all the scientific information so far available to better characterize the pivotal role of the master regulator FOXN1 transcription factor in the TEC lineage specifications and functionality.

Keywords: Foxn1 gene, TECs, thymus gland, immunodeficiency, Nude/SCID

INTRODUCTION

The thymus is the primary lymphoid organ with the unique function to produce and to maintain the pool of mature and functional T cells. This process is strictly dependent on specialized functions of thymic stromal cells (TSCs) and requires the thymus peculiar tridimensional (3D) architecture, which allows a proper intercellular cross talk (1). For a long time, the difficulty in the isolation and characterization of the thymic cellular components has limited studies on the peculiar role of individual stromal components. Novel experimental tools, including stromal cell isolation by phenotype-based cell sorting (2), dissociation and reaggregation of stromal cell subsets (3, 4), or global gene expression analysis and the evaluation of the pattern of self-antigen expression within the individual thymic epithelial cells (TECs) subset (5), allowed to acquire important knowledge on the cellular and molecular basis of thymus organogenesis and TECs functionality.

The recent discovery of disease models associated to genetic alterations of molecules implicated in thymus specification and TECs differentiation, provided new and conclusive insights regarding the pathways, the genes, and the molecular mechanism governing these processes and stromal functionality.

THE THYMUS ARCHITECTURE: REQUIREMENT OF A 3D STRUCTURE FOR A PROPER LYMPHO-EPITHELIAL CROSSTALK

The thymus provides the microenvironment essential for the development of T cells. T cell progenitors originate in the bone marrow, enter into the thymus (6, 7) and, through a series of well defined and coordinated developmental stages, differentiate, undergo selection process, and mature into functional T cells. The steps in this process are tightly regulated through a complex network of transcriptional events, specific receptor-ligand interactions, and sensitization to trophic factors, which mediate the homing, proliferation, survival, and differentiation of developing T cells (1, 8, 9).

The thymus is organized in two lobes, which are already present in mice at 21 days of thymic organogenesis and is completely organized at 1 month of post-natal life. The lobes are divided in three areas: a cortical and the dark cortical area, with a high number of lymphoid cells and epithelial cells, cortical thymic epithelial cells (cTECs); a light medullary area with a low number of mature T cells, named medullary TECs (mTECs), Hassall's bodies (HB), macrophages, dendritic cells (DCs), B lymphocytes, and

rarely myoid cells. Eventually, there is a transitional area, named cortico-medullary junction (CMJ), characterized by abundant blood vessels (10).

The unique function of the thymus in the establishment and maintenance of the T cell pool is intimately linked to this peculiar thymus architecture and to the specialized functions of the TSCs.

LYMPHO-EPITHELIAL CROSS-TALK REQUIRED FOR THYMOCYTE AND TECs DIFFERENTIATION

An important feature of the thymic microenvironment is its 3D organization, consisting of an ordered architecture of TSCs, that represents a heterogeneous mixture of distinct cell types, including cTECs, mTECs, fibroblasts, endothelial cells, DCs, and macrophages (11). Among these stromal elements, TECs are the most abundant cell types, which form a delicate 3D cellular network spanning throughout both the thymic cortex and the medulla. The requirement for the 3D-supporting stroma appears to be unique to the T cell development, as the *in vitro* differentiation program of other hematopoietic lineages, including B and NK cells, does not require a 3D structure (12).

Thymocyte development is not a cell-autonomous process, and the transition to the next stage in development relies on the proper interaction of HSCs with thymic stroma. The 3D configuration of the thymus maximizes this interaction, allowing intercellular cross-talk integral to the development of both T cells and TSCs (13). Paralleling the T cell precursor proliferation and differentiation program, immature TECs undergo a developmental sequence, resulting in the establishment of mature cTECs and mTECs organized in this 3D network. Several studies on mutant mice with an abnormal organization of thymic epithelium substantiated the concept that a reciprocal signaling between thymocytes and TSCs is required, not only for the production of mature T cells but also for the development and organization of the thymic microenvironment in a bi-directional fashion (14, 15). Mice showing a blockage of the T cell development process, in the absence of T cell receptor (TCR)-expressing cells, have a defective organization of the thymic medulla, as well (16, 17). Of note, under this condition, thymic medullary organization can be restored by the addition of mature T cells, which follows stem cell transplantation (17, 18). In adult CD3etg26 mice, lacking intra-thymic T cell precursors, a severe alteration of the cortical thymic architecture has been documented (19), even though a restoration of the architecture and TEC development in these mice can occur. Recently, the injection of either fetal or adult T-committed precursors into adult CD3etg26 mice leads to the reconstitution of thymic microenvironment, as indicated by thymocyte differentiation, organization of functional cortical and medullary areas, and generation of $Foxp3^+$ T_{reg} and $Aire^+$ mTECs (20). These data suggest that adult TECs maintain the receptivity to cross talk with thymocytes despite a prolonged absence of T cell precursors. Moreover, the absence of both thymocytes and of the 3D framework may result in changes of the keratin genes expression, thus inducing the cTECs and mTECs to undergo a de-differentiation process and to reacquire the precursor $K5^+K8^+$ cellular phenotype. Taken together, these findings suggest that signals from early $CD4^-CD8^-$ DN T cell precursors and/or their immediate progeny provide necessary signals to promote the formation of the thymic cortex, while, later in ontogeny, the differentiation of TECs into a medullary phenotype are clearly dependent on the presence of $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP) thymocytes (21–23). However, the precise molecular nature of the signals provided by developing thymocytes, which lead to the generation of the thymic stromal compartment are still incompletely defined.

Eventually, a better understanding of the developmental process through which a normal thymus structure is built, is essential for a better comprehension of the intimate mechanisms which take place within the thymus to promote the T cell development *in vivo*. This knowledge may also be useful in designing future therapeutic strategies, as alterations of the thymus structure and function may result in serious health consequences, including immunodeficiency or autoimmunity.

mTECS AND cTECS ARE SPECIALIZED CELLS PLAYING A DIFFERENT ROLE IN THE T CELL EDUCATION PROCESS

T cell ontogeny is a sophisticated process, which takes place through discrete stages during which developing thymocytes dynamically relocate in different thymic areas, following a cortico-medullary gradient.

The initial colonization of the thymus anlagen by migrant lymphoid progenitors occurs at an early stage, embryonic day 11.5 (E11.5) in mice and 8 week of gestation in humans (24, 25). Studies documented that chemokines CC ligand (CCL)21 and CCL25 play a major role in the early stage of fetal thymus colonization (26, 27). Indeed, mice deficient for these chemokines or for the cognate receptors, showed a significant reduction in the number of thymocytes compared to normal mice (28). In post-natal thymus, lymphoid progenitor cells through their cell surface adhesion molecules, such as platelet-selectin glycoprotein ligand 1, interact with P-selectin, expressed on the TECs, and thanks to this interaction they are allowed to migrate from the blood into the thymic parenchyma, in correspondence of the area around the CMJ [**Figure 1**; (29)].

Entered thymocytes started to intensely proliferate and to acquire T cell hallmarks. In this phase, T cell proliferation and differentiation are triggered by a potent combination of signals provided by cTECs. Delta-like 4 (DL4), which is an essential, non-redundant ligand for Notch1 during thymic T cell development, and IL-7 are critically involved in the activation of signaling pathways, leading to the proliferation and migration of thymocytes (30–32). In particular, these intra-thymic ligands induce the development of DN $CD25^+$ cells, which migrate toward the subcapsular region of thymic cortex (33). Several chemokine receptors have been suggested to guide the migration of immature thymocytes, such as CXCR4, CCR7, and CCR9 [**Figure 1**; (34)]. In the thymic cortex DN thymocytes begin V(D)J rearrangement of their TCRβ gene. Successfully rearranged TCRβ protein, assembled with the pre-TCRα chains, forms the pre-TCR complex. Membrane expression of pre-TCR complex, along with the Delta-Notch interaction, provides the signal necessary to induce the expression of the co-receptors CD4 and CD8, as well as V-J rearrangement of the TCRα genomic region. Subsequently, DP thymocytes with a functional TCR-αβ receptor are generated [**Figure 1**; (35)].

Thymic cortex is also the area where takes place the positive selection of DP thymocytes. Positive selection is the process by

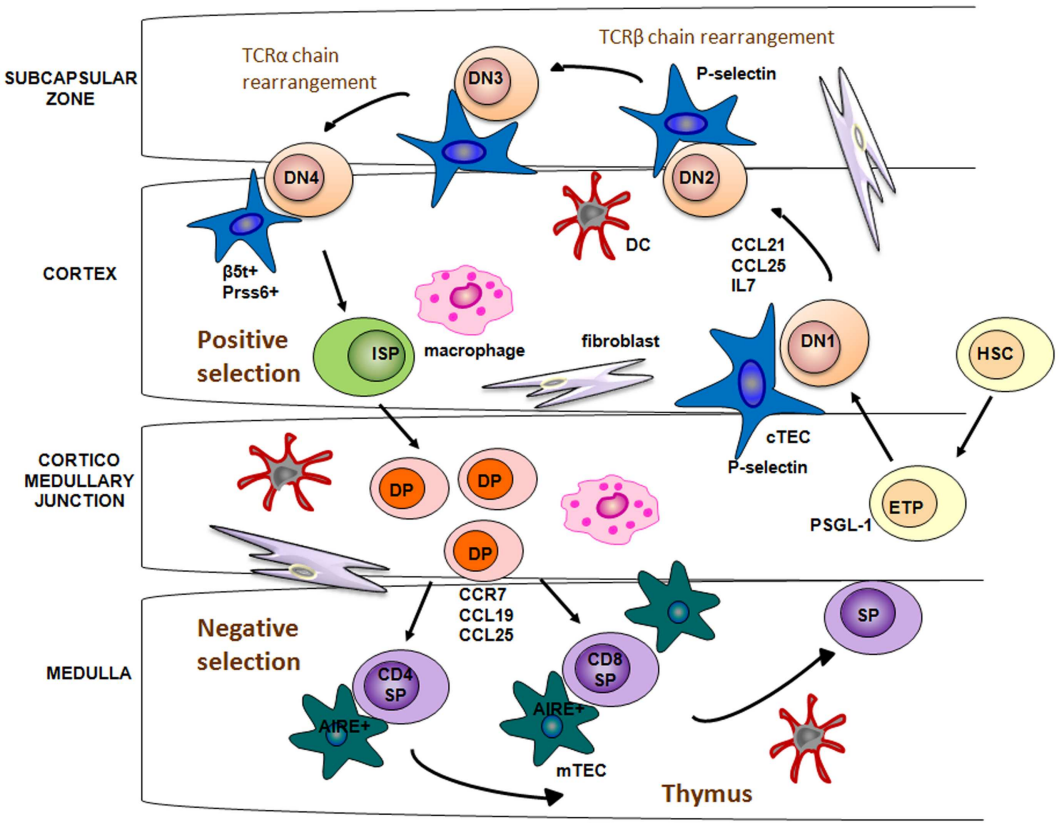

FIGURE 1 | Lympho-stromal interactions and T cell development. Bone marrow HSCs enter into the thymus through CMJ, a process mediated by CCL12 and CCL25 in the embryonic thymus and by the interaction between P-selectin and its cognate ligand PSGL-1 in adult thymus. Stimulation by IL-7 allows the relocation of DN thymocytes from the cortex to the subcapsular region. DP thymocytes bearing TCR and capable of binding to self-MHC ligands are positively selected. This process is regulated by Pssr6 and b5t, which are expressed in cTECs. Developing thymocytes are relocated from cortex to the medulla by chemotactic attraction between CCR7 and the ligands CCL19/CCL21, expressed on the mTECs. Into medulla, self-reactive thymocytes are deleted through the negative selection, a process mediated by dendritic cells and Aire-expressing mTECs.

which developing thymocytes, that recognize and bind with mild avidity peptide-major histocompatibility complex on cTECs surface, get a rescue signal through their TCR and are allowed to further maturate to the $CD4^+CD8^-$ or $CD4^-CD8^+$ SP stage. Only a small fraction (1–5%) of DP cells survive to positive selection. By contrast, the majority of DP cells, that bind with too low affinity to MHC complex, are programed to undergo death by neglect (36, 37).

Cortical thymic epithelial cells have a crucial role in the positive selection process of T cells within thymus cortex (38). Recent studies have found that cTECs exclusively express a specific form of proteasome, referred as thymoproteasome, which contains a peculiar catalytic subunit, the β5-thymus (β5t) (39). β5t subunits exhibit an unique peptidase activity, compared to other β5 subunits found in common immunoproteasome, which leads to the production of a set of self-peptides with a high affinity for class I MHC molecules (40). Moreover, β5t-deficient mice show a severe decrease in the number of $CD8^+$ SP thymocytes, but no alteration in the $CD4^+$ number or in the thymic architecture. In addition, the small fraction of $CD8^+$ T cells, positively selected by β5t-deficient cTECs, show altered immune responses toward several stimuli. Taken together these results suggest that the thymoproteasome is essential for the production of self antigens involved in the positive selection of functional $CD4^-CD8^+$ T cells (41).

As for the positive selection of $CD4^+$ T cells, two other proteins predominantly expressed in cTECs, the lysosomal protease Prss16 and Cathepsin L, have been demonstrated to be essential to generate an immunocompetent repertoire of $CD4^+CD8^-$ T cells [**Figure 1**; (42, 43)].

TCR engagement by peptide-MHC complex also triggers the expression of the chemokine receptor CCR7 in positively selected thymocytes. Thanks to the chemotactic attraction between CCR7 and its ligands, CCL19 and CCL21, expressed on the mTECs, developing thymocytes are relocated from cortex to the medulla [**Figure 1**; (44, 45)].

In order to create a repertoire of mature T cells able to recognize foreign antigens and, at the meantime, to ignore self antigens, SP thymocytes have to undergo the negative selection process in the thymic medulla. Both mTECs and DCs, play a pivotal role in this last stage of thymocyte development, which is critical to establish the central tolerance and, eventually, to prevent autoimmunity. In contrast to cTECs, mTECs are characterized by a high expression

of clustered tissue-restricted autoantigens (TSAs), the so called promiscuous gene expression (46). To date, the autoimmune regulator (AIRE) transcription factor represents the only molecule, so far identified, which contributes to the mTECs function and, in particular, to the molecular regulation of the promiscuous gene expression [**Figure 1**; (47)]. However, not all TSAs are regulated in an AIRE-dependent manner, suggesting that other molecular mechanisms, such as epigenetic mechanisms, may be involved in mTECs function regulation. TSAs associated with class II MHC molecules are presented directly by mTECs or indirectly by DCs to developing thymocytes (48). T cells which recognize with a high avidity self antigens are deleted. Remarkably, only a few number of mTECs express a given TSAs (about 50–500 per thymus), and lead to apoptosis by negative selection of a few thymocytes (37, 49, 50). A possible explanation is that the high motility of thymocytes within the thymic medulla during a period of 4–5 days, allows each of them to interact with mTECs (51). DCs play a similar role in the negative selection process. They are attracted in the thymic medulla by the chemochine XCL1 (lymphotactin), produced by mTECs in an AIRE-dependent manner. Differently from mTECs, DCs are not able to produce TSAs and the TSAs expressed mostly derive from the phagocytosis of apoptotic mTECs (52, 53). mTECs and DCs not only contribute to the establishment of central tolerance through the deletion of self-reactive T cells, but, also, through the generation of regulatory T cells (T_{regs}) (54, 55, 65, 153), which act in the periphery by suppressing autoreactive T cells, which have escaped to the process of the central tolerance.

A body of evidence documents that the expression of an autoreactive TCR leads to the entry of the thymocyte into the T_{reg} lineage. T_{regs}, that are about 5–10% of peripheral T cells $CD4^+$, constitutively express the CD25 molecule and share several immunological features, in humans and mice (56, 57). These cells specifically express the transcription factor FOXP3 (Foxp3 in mice) that plays a pivotal role in T_{regs} differentiation and function (58). The Foxp3 promoter region and the conserved non-coding sequence 2 (CNS2) (known as TSDR, the T_{reg}-specific-demethylated-region) are fully methylated in immature thymocytes (59, 155). At the beginning of T_{reg} development, an appropriate TCR/CD28 signal is needed to make available the *Foxp3* promoter through shift of the Protein Inhibitors of Activated STAT 1 (PIAS1), a signal cascade, which results in the NF-κB-mediated transcription of genes playing a role in T_{reg} differentiation (60, 61).

THYMIC FORMATION: NEW INSIGHTS IN EPITHELIAL LINEAGES SPECIFICATION

In the mouse, mTECs and cTECs originated from the third pharyngeal pouch endoderm and the thymus anlage are located next to that of the parathyroid. The expression of Forkhead-box transcription factor n1 (Foxn1) approximately at E11.5 is crucial for the subsequent epithelial differentiation, since in its absence, the colonization of the anlage by T cell progenitors from the bone marrow fails (62) and the subsequent T cell development and TECs formation is aborted, resulting in a severe immunodeficiency (63, 64, 66, 154).

The maturation process of TECs during thymic organogenesis could be divided in two genetic phases. The first stage is independent from the *Foxn1* expression and consists in the induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, through the expression of genes including the *Eya1* and *Six* (67), *Hoxa3* (68), and *Tbx1* (69, 70). During the second genetic phase, epithelial patterning and differentiation take place and the *Foxn1* expression drives the immature epithelial cells to differentiate into functional cTECs and mTECs (71).

FOXN1-INDIPENDENT GENETIC STAGE OF TEC DIFFERENTIATION

In the first phase of the thymus organogenesis an interaction between epithelial and mesenchymal cells occurs, while at the later phase lympho-epithelial interaction predominates (72). In mice, at about E10.5 the mesenchymal cells are able to respond to the endodermic signals, which induce the development of the primordial thymic epithelium (73, 74). Subsequently, at about E12.5, the thymic rudiment is colonized by progenitors come from the fetal liver, thus resulting in a tight epithelial-thymocyte interaction within the mesenchymal derived capsule. This thymic rudiment contains the $EpCam^+Plet1^+$ epithelial population (72, 75), which includes a common thymic epithelial precursor (TEPC), from which both cTECs and mTECs will be subsequently generated (72, 76).

Through studies on animal models carrying molecular alterations of distinct genes, the key role of several transcription factors involved in the thymus organogenesis and TEC-sublineage specification process, have thus far been identified (77). In particular, several genes, including *Tbx1* (69, 70), *Pax1*, *Pax3*, *Pax9* (78–80), *Hoxa3* (68), *Eya1*, and *Six1* (67) have been shown to play a central role in the thymus ontogeny. Indeed, their molecular alteration affects this well-orchestrated process, leading to disruption of the thymic architecture. Abnormalities of the paired box (Pax) family transcription factors Pax1 or Pax9 result in a blockage of the thymus organogenesis (79, 81). Mutations in the Hox transcription factor family member, Hoxa3, expressed on both thymic epithelium and mesenchymal cells, result in athymia (68). Furthermore, the homozygous loss of *Tbx1*, related to the DiGeorge syndrome phenotype, leads to thymic a/hypoplasia in humans (69, 82), while mice heterozygous for a null allele of *Tbx1* show a mild phenotype without thymus anomalies (83). Therefore, the expression of *Tbx1* both in the pharyngeal core mesoderm and in the pharyngeal endoderm is required for a proper thymus development. However, it remains to be elucidated whether the expression of *Tbx1* in the TECs occurs and whether the gene participates in the TECs development (4).

FOXN1-DEPENDENT GENETIC STAGE OF TEC DIFFERENTIATION

In both humans and mice, the primordial TECs are yet unable to fully support T cell development and only after the transcriptional activation of the *FOXN1* gene in the thymic epithelium this essential function is acquired. *FOXN1* is a master regulator in the TEC lineage development in that it promotes down-stream the transcription of genes implicated in the thymus organogenesis and TECs full differentiation.

Forkhead-box n1 transcription factor belongs to the FOX transcription factor family implicated in a variety of biochemical and cellular processes, including development, metabolism, aging, and cancer (84, 85). During the post-natal life, *Foxn1* is selectively

expressed only in thymic and skin epithelia, where it regulates the expression of several molecular targets to maintain the balance between growth and differentiation (86, 87). The signals required for *FOXN1* expression, and its activity, are still unclear, even though the wingless (Wnt) proteins (88) and bone morphogenetic protein (BMP) signaling have been shown to regulate *FOXN1* expression (89). Even though the complete pattern of *FOXN1* expression over the time and its role are not yet completely defined, studies on mouse and human model of gene alterations enormously helped unravel important issues on its role. Mutations in *Foxn1* gene lead to alymphoid cystic thymic dysgenesis due to a defective TECs differentiation process (63, 90). In both mice and humans *FOXN1* abnormalities lead to a hairless phenotype (87, 154).

In the *Foxn1*-dependent step of thymus organogenesis, precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bi-potential TEC progenitor (4, 72, 76). It has been reported, that *Foxn1* is differentially expressed during the TE-lineage specification, since it is expressed in all TECs during the pre-natal life, but not in all TECs postnatally, indicating that the gene is highly developmentally regulated. There is a body of evidence documenting different effects of *Foxn1* expression in mTEC and cTECs. Particularly, studies on K5- and K18-CreERT-mediated *Foxn1*-deleted mouse models suggested that during the post-natal life, the loss of *Foxn1* affected mTECs, characterized by the expression of K5 and K14 keratins type. Conversely, the loss of *Foxn1* did not affect cTECs, which express the keratins K8 and K18 (91, 92). Taken together, these data suggest that cTECs and mTECs are not equally *Foxn1*-dependent in the post-natal life.

Recent reports highlighted a central role for *Foxn1* in TECs homeostasis in the adult thymus and its necessary role for the functionality and survival of adult TEC progenitors (92), expressing $K5^+$ and $K14^+$ markers. This role in adult thymus seems to be exerted in cooperation with other stem cell-related genes, such as *p63*. Of note, the transcription factor p63, encoding for multiple isoforms (93), plays a pivotal for the development of stratified epithelia of several tissues, such as epidermis, breast, prostate, and thymus (94). In the thymus, the p63 protein drives the proliferation of epithelial progenitor cells (94, 95). Therefore, it has been hypothesized that *p63* and *Foxn1* could act synergistically through the formation of a *p63-Foxn1* regulatory axis aimed at regulating TECs homeostasis. However, the molecular mechanism through which the proliferation regulator *p63* and differentiation regulator *Foxn1* collaborate in this axis are still unclear.

FOXN1-MEDIATED GENE EXPRESSION FOR TEC DIFFERENTIATION

Forkhead-box n1 is directly or indirectly implicated in the transcriptional regulation of a panel of genes involved in thymus development and function.

Pax1 is a key regulator of TEC differentiation/survival balance. *Pax1* is expressed in the third pharyngeal pouch from E9.5 during the thymus ontogeny, while in the post-natal thymus only in cTEC (96). Even though the regulation of *Pax1* is still unclear, from E11.0 its expression requires *Hoxa3* (68). Of note, the loss of *Hoxa3* impairs the intrinsic ability of the neural crest cell population to differentiate and/or to lead to the differentiation of the tissues of pharyngeal arch and pouch. Indeed, in *Hoxa3* mutant mice the thymus is absent and thyroid hypoplasia has been documented (68). Moreover, the first step of thymus development is the expansion of mesenchymal neural crest in the posterior part of the third pharyngeal pouch. Prior to this event, in the *Hoxa3* mutant embryos a marked reduction in *Pax1* expression has been shown. Similarly, *Pax1* mutant mice also show thymic hypoplasia, suggesting a role for *Hoxa3* in maintaining *Pax1* expression in these cells (68). In the thymic primordium, *Pax1* expression is under the control of *Foxn1* (71). This finding indicates that *Foxn1* and *Hoxa3* are both involved in the network of molecular signals that regulates *Pax1* expression, thus demonstrating the existence of a molecular and/or functional interaction between *Hoxa3* and *Foxn1* [**Figure 2**; (71)]. In keeping with this, $Hoxa3^{+/-}Pax1^{-/-}$ compound mutant mice display a few phenotypic hallmarks of the $Foxn1^{R/R}$ mouse model, which expresses low-dose of *Foxn1*, such as hypomorphic post-natal thymus, and reduced levels of MHC class II expression on the TECs surface (80). These data suggest two alternative hypothesis: *Hoxa3* may regulate *Foxn1*, which, in turn, regulates *Pax1* expression in the thymic primordium, in a *Foxn1*-dependent manner, or *Hoxa3* and *Foxn1* induce *Pax1* expression in the third pharyngeal pouch and in early thymus primordium.

It has also been shown that *Foxn1* regulates the expression of *CCL25* and *Dll4* (**Figure 2**). These genes play a pivotal role in the thymocyte development, since *CCL25* regulates the colonization of the fetal thymus (97), while the Notch ligand *Dll4* is involved in the commitment of hematopoietic progenitors to the T cell lineage (30). In both early fetal TEC and in the post-natal thymus, *Dll4* expression is directly related to the *Foxn1* expression (71). Furthermore, these molecules are absent in the *Foxn1* null thymus, even though there is evidence indicating that their expression may occur in a *Foxn1*-independent manner in TECs (98, 99). Eventually, in a recent report it has been shown that *Foxn1* is upstream of dll4a and ccl25a expression in *medaka fish*, thus confirming the relationship with this transcription factor (100).

THE HUMAN NUDE/SCID PHENOTYPE: A MODEL OF THYMIC MICROENVIRONMENT DISRUPTION AND FAILURE OF THE T CELL DEVELOPMENT

The Nude/severe combined immunodeficiency (SCID) phenotype represents the prototype of thymic architecture disruption due to alterations of the *FOXN1*, which is the master regulator of TE-lineage specification (71).

In humans, as in mice and rats, mutations in the "nude" *Foxn1* gene induce the hairless phenotype, associated with a rudimentary thymus gland (T cell related primary immunodeficiency). The human Nude/SCID phenotype (MIM 601705; Pignata Guarino Syndrome) was first identified in 1996, after more than 30 years from the initial mouse description, in two sisters originated from a small community with a high grade of inbreeding, who showed congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T cell immunodeficiency, inherited as an autosomal recessive disorder (154). This phenotype was associated with a C792T transition in the *FOXN1* gene, which resulted in the nonsense mutation R255X in the exon 4 (formerly exon 5), with a complete absence of a functional protein similar to the previously described rat and mouse *Foxn1* mutations (101–103).

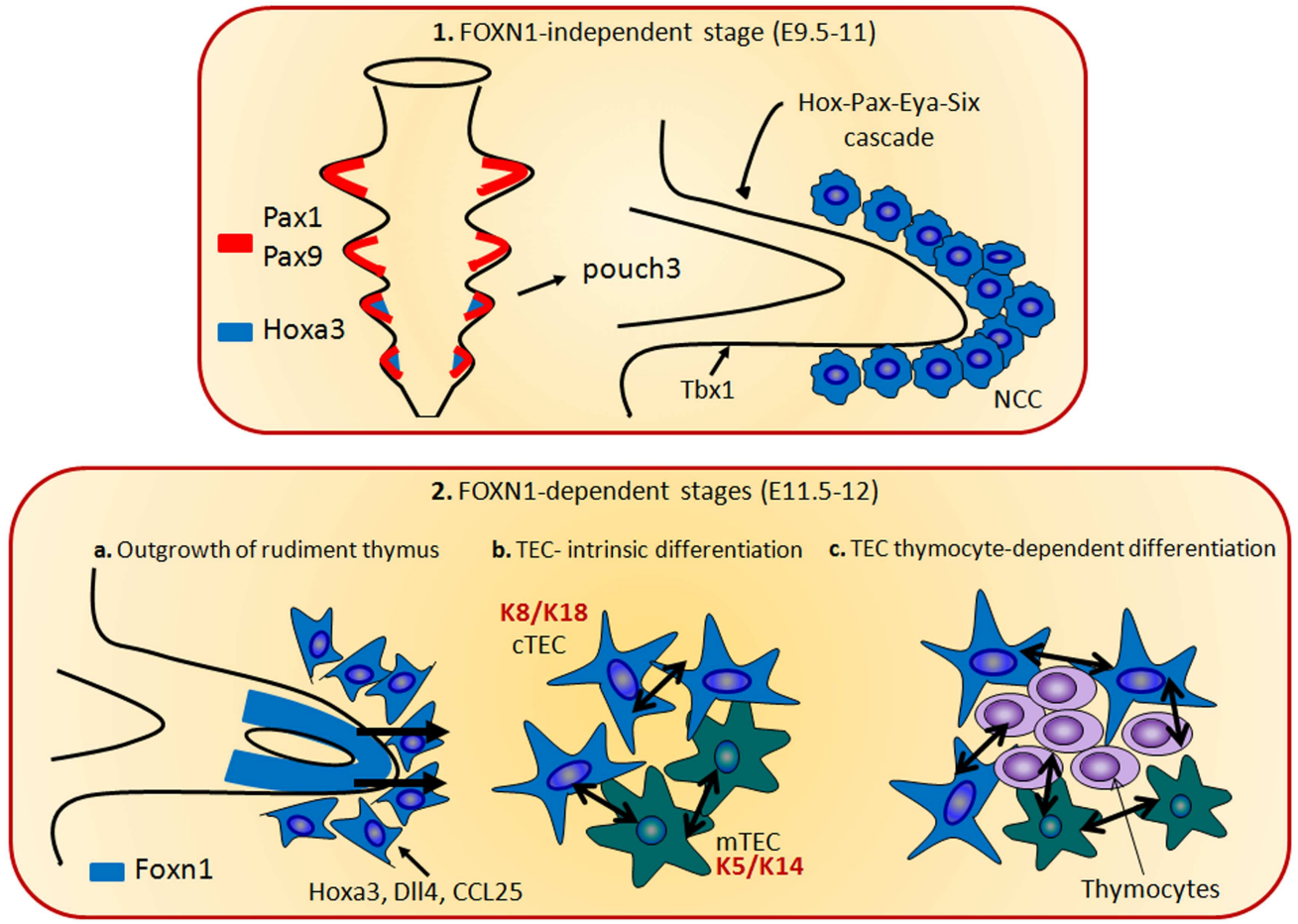

FIGURE 2 | The thymus organogenesis. The thymus organogenesis is characterized by two genetic phases. The first stage is independent from the *Foxn1* expression (1) and consists in the induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, through the expression of genes including the *Eya1*, *Six*, *Hoxa3*, and *Tbx1*. *Hoxa3* and *Eya1* are also required in neural crest cells (NCCs). In the phase 2, *Foxn1* regulates the expression of *CCL25*, *Dll4*, and *Hoxa3*, necessary for the thymocytes and TECs differentiation. During this step, cTECs (expressing K8 and K18 keratin type) and mTECs (expressing K5 and K14 keratin type) originate from the same bi-potential TEC progenitor. The crosstalk between TECs and developing thymocytes is required to generate fully mature TECs and functional T cells.

In the absence of *Foxn1* expression, thymic development is halted at a rudimentary stage. As a consequence, in the affected patients the thymic lobe is still present but intra-thymic lymphopoiesis is completely blocked (63, 104) leading to severe primary T cell immunodeficiency (105–107) and to death in early childhood from severe infections (105, 108–112, 154). *Foxn1* is also involved in morphogenesis and maintenance of the 3D thymic micro-structure, which is necessary for a fully functional thymus (113, 114). In fact, evidence is available that in an *in vitro* 2D culture system consisting of a monolayer of mouse bone marrow stromal OP9 cells it is possible to generate mature T cells, only if these cells are transduced with the Notch ligand Delta-like 1 (OP9-DL1) (115, 116), whose pathway exerts a pivotal and necessary role in promoting the induction of T cell-lineage commitment (117–119). Of note, in all these co-culture systems, the stromal cells are enforced to overexpress Notch ligands, and their expression by TECs seems to be maintained only in a 3D thymus structure (120). In human Nude/SCID, the T cell defect is characterized by the absence of proliferative response to the common mitogens and a severe blockage of the T cell differentiation (154). Recent studies revealed the presence of some circulating T cells of non-maternal origin in patients carrying alterations of *FOXN1* gene. These cells have been shown to be predominantly double-negative αβ T cells ($CD3^+CD4^-CD8^-$, DN) and to exhibit a regulatory like T cell phenotype ($FoxP3^+$). This finding raised important issues regarding the site of differentiation of these cells. One hypothesis is the persistence of a thymic rudiment, which allows a partial T cell development (109). Alternatively, a T cell differentiation, even though partial and ineffective to result in a productive immunity, could occur at an extra-thymic site. In both pre-natal and post-natal life, the TCRBV spectratype repertoire in Nude/SCID patients is oligoclonal, thus confirming the immaturity of the process and, at the same time, that developmental events do take place at some extent (111, 112).

For many years, the human counterpart of the nude mouse phenotype has been erroneously considered the DiGeorge syndrome,

which occurs spontaneously and is mainly characterized by thymic hypo/aplasia and a mild T cell defect. However, several lines of evidence argue against the analogy between these two disorders. In fact, the DiGeorge syndrome is often associated with neonatal tetany and major anomalies of great vessels. These defects are due to malformation of the parathyroid and heart, derived from a major embryologic defect in the third and fourth pharyngeal pouch from which the thymus primordium emerges. In addition, in this syndrome hairlessness is missing and gross abnormalities of skin annexa are not found. Children with DiGeorge syndrome may also have lymphopenia, with a mild reduction of T cells, that are however usually responsive to common mitogens.

In Nude/SCID patients, skin is tighter than usual and is characterized by basal hyperplasia and dysmaturity. Alopecia is primitive in nature, in that it can be observed at birth and persists after bone marrow transplantation, thus ruling out the acquired nature of the disorder. In keeping with this, in athymic mice, completely lacking body hair, restoration of the thymus did not lead to hair growth, indicating a direct participation of FOXN1 to hair follicle development (87). The most frequent phenotypic alteration affecting the nails is koilonychia ("spoon nail"), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; canaliform dystrophy and a transverse groove of the nail plate (Beau line) may also be observed (121). However, the most specific phenotypic alteration is leukonychia, characterized by a typical arciform pattern resembling a half-moon and involving the proximal part of the nail plate. These alterations of digits and nails were also reported in a few strains of nude mice. Of note, nail dystrophy has also been observed in heterozygous subjects carrying *FOXN1* alterations (121). *FOXN1* is known to be selectively expressed in the nail matrix, where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygosity for this molecular alteration (121).

Autoptical study of a fetus homozygous for R255X mutation revealed multiple-site neural tube defects, including anencephaly and spina bifida. This finding may help explaining the high rate of mortality *in utero* observed in the population where the first patients were identified (105). Intriguingly, the other forms of SCID become clinically evident only during the postnatal life, when the protection of the newborn transferred from the mother immune system declines. This observation, suggests that other causes different from immunodeficiency, are responsible for the high rate of mortality *in utero* and led to consider the Nude/SCID mutation and anencephaly causally related. Of note, in a recent study, the mouse Foxn1 gene was found to be expressed also in epithelial cells of the developing choroids plexus, a structure filling the lateral, third and fourth ventricles of the embryonic brain (105). Moreover abnormality in the development of corpus callosum were also found in another FOXN1 mutated fetus even in the absence of anencephaly, indicating that the transcription factor may play a role as a co-factor in the brain ontogeny (105).

Altogether these findings suggest that FOXN1 may also be implicated as co-factor in the development of vital systems required for a proper fetus development, thus explaining the mortality in the first trimester in fetuses carrying the genetic alterations, which is not justified by the SCID *per se*.

FOXN1 MUTATION PREVENTS THE PRE-NATAL T CELL DEVELOPMENT IN HUMANS

It is now clear that FOXN1 acts as a transcription factor implicated in the differentiation of thymic and skin epithelial cells, even though many of its molecular targets still remain to be discovered. Most of the knowledge so far available has been achieved in humans in the post-natal life, while little is known about FOXN1 role during the pre-natal life.

Of note, other FOX family members, including *Foxq1* and *Foxm1b*, are important during embryogenesis, being involved in a variety of biological processes (122). Approximately 50% of *Foxq1*$^{-/-}$ murine embryos die *in utero*, thus suggesting the requirement of this gene during embryogenesis (123). Similarly, *Foxm1b* is important during liver regeneration (124).

Studies on thymus organogenesis revealed that *Foxn1* is expressed in all TECs during fetal stages. Of note, *Foxn1*$^{-/-}$ mice showed undifferentiated TECs responsible for a blockage of thymopoiesis and severe immunodeficiency (125). Recently, the identification of a human *FOXN1*$^{-/-}$ fetus gave the unique opportunity to study in humans the T cell development *in utero*, in the absence of a functional thymus. Vigliano et al. documented a total blockage of the CD4^{+} T cell maturation and a severe impairment of CD8^{+} cells, with an apparent bias toward TCRγδ^{+} cells (112). In this case in the congenital absence of the thymus was due to R255X missense mutation in the *FOXN1* gene. In particular, it has been reported that in the absence of FOXN1 a few not functional CD8^{+} cells, mostly bearing TCRγδ in the absence of CD3, presumably of extra-thymic origin could develop in both humans and mice (126–128). Further analysis of the fetal RNA, performed to evaluate the variable-domain β-chain (Vβ) families' usage among T lymphocytes, revealed that the generation of TCR diversity occurred at some extent in the *FOXN1*$^{-/-}$ fetus, but was abnormal. Thus, these data provided a further evidence of the crucial role for FOXN1 in the early pre-natal stages of T cell development and not in the B and NK-cell differentiation, these populations being normally present in the Nude/SCID fetus (112). A similar impairment of the T cell differentiation with a selective blockage of CD4 differentiation but not of CD8, was detected in murine models characterized by the absence of the nuclear high-mobility group (HMG) box protein TOX (107).

The identification of a limited number of CD8^{+} cells bearing the TCRγδ suggests that this cell population may develop at extra-thymic sites in a *FOXN1*-independent manner, even though they are unable to sustain a productive immune response into the periphery. Indeed, evidence exists indicating that T cells may also differentiate at extra-thymic sites, as intestine and liver (129–133). Of note, the majority of thymus-derived T lymphocytes bears the αβ chains of TCR and a few of them express the γδ heterodimer (134), while the T cell pool developed outside the thymus is characterized by a higher proportion of TCRγδ^{+} T cells expressing the CD8αα homodimer, instead of the CD8αβ (135, 136). Moreover, also DN T cells (CD3^{+}CD4^{-}CD8^{-}) and lymphocytes expressing CD7 and CD2 in the absence of CD3 (CD2^{+}CD3^{-}CD7^{+}) are generally considered of extra-thymic origin (135–137).

In spite of the well documented knowledge on the role of the primary lymphoid organ to foster T cell development, some still unsolved issues in human athymic conditions indicate that an in-depth information of the overall process is still to be achieved and, in particular, the involvement of different tissues in T cell ontogeny must be definitively clarified. Since FOXN1 is selectively expressed in the thymus and skin, one possibility to explain the presence of the few non-functional $CD8^+TCR\gamma\delta^+$ cells in Nude/SCID fetus is that skin epithelial cells could play a partial role in T cell ontogeny, as already shown in *in vitro* models (138, 139).

THYMUS TRANSPLANTATION: A PROMISING TREATMENT TO ATHYMIC DISORDERS

Forkhead-box n1 deficiency is a very rare immunodeficiency with unfortunately poor chance of curative treatments. Recently, thymus transplantation has emerged as a promising treatment for children affected with congenital athymia (140–143), as that observed in complete DiGeorge anomaly and in *FOXN1* deficiency. Conceptually, the thymus transplant seems to be in principle the more appropriate therapeutic strategy, taking into account that bone marrow transplantation performed in one child with *FOXN1* deficiency, failed to induce a long-term sustained immune reconstitution. In particular, in this patient no reconstitution of the naïve T cell pool was observed (144).

Thymus transplantation has been first used in children affected with complete DiGeorge anomaly, with excellent clinical and immunologic results (141). In order to achieve immune reconstitution, cultured post-natal allogeneic thymus tissue slices were transplanted into the quadriceps muscles of the athymic host (145). The migration of host bone marrow stem cells to the donor graft allow them to develop into naive T cells, which then emigrate out of the engrafted thymic tissue into the peripheral blood. Thymopoiesis is observed in biopsies of the transplanted thymus within 2 months of transplantation (140) and naive T cells are detected in the peripheral blood approximately 3–5 months after transplantation (146, 147). Taking advantage from this previous experience, a few years ago an allogeneic thymus transplantation has been used for the first time in two unrelated infants with Nude/SCID phenotype due to a deficiency of the transcription factor FOXN1 (111). The clinical phenotype of the two subjects was characterized by the absence of naïve T cells, total alopecia, nail dystrophy, and severe infections, as disseminated *Bacillus* Calmette–Guérin in subject 1 and severe respiratory infections in subject 2. Molecular analysis, performed to confirm the clinical suspect of the Nude/SCID phenotype, revealed the presence of a homozygous R255X mutation in the *FOXN1* gene in subject 1, the same of that previously described (107), and a homozygous R320W novel missense mutation in the subject 2. Moreover, subject 1 showed, like a small percentage of complete DiGeorge patients, referred as atypical complete DiGeorge, circulating oligoclonal T cells of non-maternal origins, which were predominantly double-negative T cells, and a T cell proliferative response to PHA within the normal range. Because of that, before thymus transplantation subject 1 have required immunosuppression regimen to prevent graft rejection. Differently, immunosuppression was not used for the subject 2, who had, like typical complete DiGeorge patients, very few T cells (141, 146).

Results obtained with thymus transplantation were encouraging in both FOXN1-deficient subjects, and led to a full T and B cell reconstitution and functional rescue. Indeed, both subjects developed naïve T cells, diverse TCR repertoires and an *in vitro* proliferative T cell responses against different antigens. Eventually they reached normal serum Ig levels with generation of protective antibody specific titers. Of note, HLA matching for class I and II did not seem to interfere with T cell counts after thymus transplantation, being subject 2 transplanted without any HLA matches. However, $CD8^+$ T cell number, although apparently functional, was disproportionally low compared to $CD4^+$ T cells (111). A poor CD8 recovery has also been described in complete DiGeorge patients, who underwent HLA-mismatched thymic transplantation (141, 148). Possible explanations are that the phenomenon is related to the HLA mismatch between host hematopoietic precursors and allograft thymic epithelia or to alterations in the thymic graft due to transplantation procedures.

Functionality of the thymic allograft has been assessed for the first time through signal joint (sj) and DβJβ T cell receptor rearrangement excision circle (TREC) analyses (109). The sj/βTREC represents a ratio between early and late products of TCR rearrangements, which directly correlate with thymic output and provide an indirect measurement of thymocyte division-rate (149–151). The sj/βTREC ratio quantification, conducted in subject 1 with R255X mutation, was very low during the peri-transplant period and comparable to those observed in healthy children at 2.5 years post-transplant. Of note, 4 years post-transplantation a decrease of sj/βTREC ratio associated with a reduction in sjTREC levels and in the number of naïve cells were found, suggesting the decline in thymic allograft output (109). This decline might be due to the reduced longevity of the thymus allograft or to peripheral homeostasis of the T cell pool maintenance following its replenishment. Overall, the thymus transplantation seems to be a promising curative strategy for subjects with athymia due to *FOXN1* deficiency or complete DiGeorge syndrome in the perspective of long-term clinical benefit.

CONCLUSION

The integrity of the thymic epithelial architecture allows the growth, the differentiation, and TCR repertoire selection of immature T cells, thus originating fully mature and functional T cells. Of note, the failure to generate or to maintain the proper 3D thymic architecture leads to severe immunodeficiency or autoimmunity. The unique function of the thymus in the establishment/maintenance of the T cell pool is related not only to the peculiar 3D structure, but also to the specialized functions of the thymic stroma. Indeed, lympho-stromal interactions within the multicellular thymic microenvironment play a crucial role in the regulation of the T cell development. Moreover, these interactions are based on a bilateral crosstalk between stromal cells and traveling thymocytes, which, in turn, are able to provide important signals for the TECs differentiation.

Thymus organogenesis and T cell development are sophisticated biological processes, which require the activation of a wide panel of genes. There is evidence that the master regulator of the thymus development is the *Foxn1* gene, since it is required at

multiple intermediate stages of the TE-lineage specification either in the fetal and adult thymus, through the direct or indirect regulation of genes involved in the thymus development and function. These genes include *Pax1*, *Hoxa3*, *CCL25*, *Dll4*, *p63*.

Studies on the animal and human model of the Nude/SCID phenotype have provided an enormous contribution in identifying the crucial role of *Foxn1* to drive the thymus development, even though many issues regarding the transcriptional regulation of the TECs specification and homeostasis still remain to be solved. The development *in vitro* of cellular models of TEC lineage differentiation, by using the technology of nuclear reprograming, will be certainly useful to better characterize the discrete stages of the TECs differentiation and the molecular mechanism involved in the process.

Eventually, the *in vitro* re-build of a thymic environment capable to reproduce tissue features of primary lymphoid organs (139, 152) could be a promising and valuable tool for the treatment of congenital athymia, including *FOXN1* deficiency, along with the thymus transplantation, which is emerged as a potential treatment for these disorders.

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Which model better fits the role of Aire in the establishment of self-tolerance: the transcription model or the maturation model?

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The discovery of Aire-dependent transcriptional control of many tissue-restricted self-antigen (TRA) genes in thymic epithelial cells in the medulla (medullary thymic epithelial cells, mTECs) has raised the intriguing question of how the single *Aire* gene can influence the transcription of such a large number of TRA genes within mTECs. From a mechanistic viewpoint, there are two possible models to explain the function of Aire in this action. In the first model, TRAs are considered to be the direct target genes of Aire's transcriptional activity. In this scenario, the lack of Aire protein within cells would result in the defective TRA gene expression, while the maturation program of mTECs would be unaffected in principle. The second model hypothesizes that Aire is necessary for the maturation program of mTECs. In this case, we assume that the mTEC compartment does not mature normally in the absence of Aire. If acquisition of the properties of TRA gene expression depends on the maturation status of mTECs, a defect of such an Aire-dependent maturation program in Aire-deficient mTECs can also result in impaired TRA gene expression. In this brief review, we will focus on these two contrasting models for the roles of Aire in controlling the expression of TRAs within mTECs.

Keywords: autoimmunity, thymic epithelial cell, self-antigen, gene transcription, cell differentiation

The current prevailing view regarding the role of Aire in self-tolerance is that it is involved in the transcriptional control of many tissue-restricted self-antigen (TRA) genes in medullary thymic epithelial cells (mTECs) (1). In other words, TRAs are considered to be the direct target genes of Aire's transcriptional activity (the transcription model) (2). This view was first suggested in a paper reporting that Aire-deficient mTECs showed dramatically lower expression of TRAs than wild-type mTECs (3). Since this landmark report, the transcription model has prompted many studies of Aire in an attempt to clarify how the single *Aire* gene can influence the transcription of such a large number of TRAs within mTECs (4–7). Unfortunately, to obtain a mechanistic insight into this interesting phenomenon, it has been necessary to employ cultured cells transfected with the *Aire* gene, because the fraction of naturally Aire-expressing mTECs in the thymic stroma is too small to work with. However, no appropriate cultured cell lines that could be used reliably in place of Aire-expressing mTECs *in vivo* have been available. Nonetheless, overexpression of Aire in cultured cells resulted in increased transcription of many TRAs, in accordance with the transcription model. However, it is important to pay more attention to the uniqueness of bona fide mTECs *in vivo*, which show characteristics very different from those of cultured cell lines; although several cell lines derived from the thymic stroma are available for both humans and mice, none of them show typical characteristics of mTECs such as high expression of FoxN1, MHC class II, and CD80, even though they are positive for cytokeratin and epithelial cell markers (e.g., keratin 5, EpCAM, and lectin UEA-1 binding). Most importantly, none of these cell lines express a reliable level of Aire at the protein level. However, large aspects of Aire's action on TRA gene expression in the transcription model were deduced on the basis of the effects of lack of Aire expression in mTECs *in vivo* (i.e., the phenotypes of Aire-deficient mice) and the opposite effects of Aire overexpression in mTEC-"like" cells *in vitro* (i.e., in transfection studies). The reality is that there is still a fairly wide gap between these two experimental settings that needs to be bridged.

In comparison with the remarkable changes noted in the expression profiles of TRA genes in Aire-deficient mTECs, morphological alterations in the medullary components from Aire-deficient mice were not initially appreciated until Farr's paper had appeared (8). This was the main reason why insufficient attention was paid initially to another proposed explanation for the reduced TRA gene expression in Aire-deficient mTECs: the maturation model (2). However, fairly recent detailed studies of Aire-deficient thymi have revealed several important aspects of the Aire-dependent differentiation programs of mTECs, such as increased numbers of mTECs with a globular cell shape (8, 9), contrasted with reduced numbers of terminally differentiated mTECs expressing involucrin, the latter being associated with reduced numbers of Hassall's corpuscles (9, 10). Although not fully investigated, increased percentages of mTECs expressing high levels of CD80 ($CD80^{high}$) is another suggested aspect of the Aire-dependent mTEC differentiation program (11–13). In this regard, it is noteworthy that the Aire-dependent mTEC differentiation

program can be linked to the control of TRA gene expression, in which the role of Aire may be perceived from a different viewpoint. Given that acquisition of the properties of TRA gene expression depends on the maturation status of mTECs (14, 15), any defect in such an Aire-dependent maturation program could also account for defects of TRA gene expression in Aire-deficient mTECs. In such a case, TRA genes would not have to be the direct target of Aire. Instead, Aire-deficient mTECs would have defective TRA gene expression, because they are not fully differentiated to stage(s) where other undetermined transcriptional means for TRA gene expression beyond Aire become available and/or active (**Figure 1**). Having said so, the exact point in the differentiation process at which Aire-deficient mTECs are prevented from differentiating further still remains unclear. Investigation of this issue has been hampered by the current lack of suitable markers for the mTEC differentiation program: so far, CD80 and MHC class II remain the few that are available. Precise elucidation of the target gene(s) relevant to the progression of mTEC differentiation controlled by Aire is an essential task to support the maturation model.

Currently, there is no firm evidence to support either model for Aire's role in the control of TRA gene expression. Therefore, there is a need to develop better *in vivo* experimental systems for investigating this issue. In this connection, the results obtained by examining *Aire* gene expression under control of the rat insulin promoter using transgenic mice merit attention (16). It was found that alterations of the transcriptome did not mirror those created by abrogation of Aire within mTECs. This may not be surprising, but it is nevertheless important: if cell types differ, the effects of functional gain or loss of a transcription factor, such as Aire, can in turn differ markedly. This is especially important in the case of Aire, since mTECs are unique in showing promiscuous gene expression and a heterogeneous composition (17, 18). Thus, it cannot be over-emphasized that the roles of Aire need to be studied using *in vivo* models, and not *in vitro* systems using mTEC-surrogate cells.

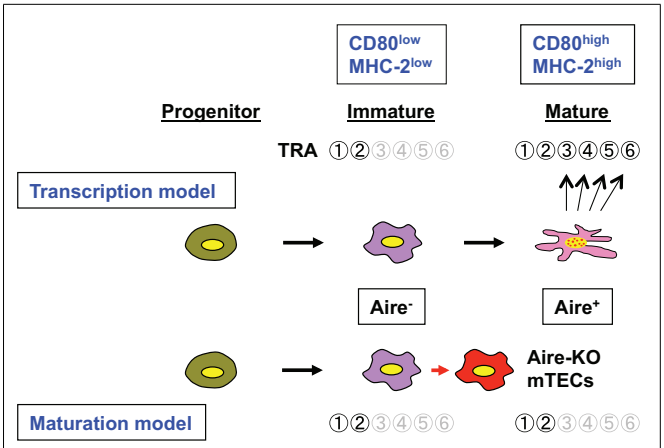

FIGURE 1 | Schematic representation of the roles of Aire in TRA gene expression associated with the mTEC differentiation program. Aire-expressing mTECs develop from their progenitor via an immature stage (CD80^{low}MHC-2^{low}) where only limited numbers of TRAs (e.g., ① and ②) are expressed. Aire is expressed at the mature stage (CD80^{high}MHC-2^{high}), and the transcription model (upper) suggests that Aire directly activates many target TRA genes within mature mTECs (so-called Aire-dependent TRAs; ③–⑥). Consequently, lack of Aire results in defective expression of Aire-dependent TRA genes. In contrast, the maturation model suggests that Aire plays an important role in the maturation program of mTECs, and that expression of Aire-dependent TRA genes, such as ③–⑥, can be accomplished in terminally differentiated mTECs showing a dendritic to fibroblastic morphology that have fully matured with the help of Aire. According to this scenario, lack of Aire causes a halt in mTEC maturation at a stage where promiscuous gene expression in the cells is still not possible (marked as "Aire-KO mTECs"); lack of Aire in mTECs results in premature termination of differentiation, and these CD80^{high} Aire-less mTECs have a more globular cell shape and lack the transcriptional machinery for Aire-dependent TRA genes (lower). Because Aire-independent TRA genes, such as ① and ②, can be expressed before the mature stage, lack of Aire has little impact on their expression.

Regardless of the models employed, there are several important issues related to the molecular regulation of TRAs within mTECs in the context of Aire. Obviously, the transcriptional control of TRAs is different from the regulation seen in their authentic tissues, as exemplified by differences in the transcriptional start sites of individual TRA genes (19). Although the fact that transcriptional hierarchies driving the development of the pancreas and transcription of the *insulin 2* gene are not maintained in mTECs (19, 20) might be more consistent with the transcription model, it does not contradict the maturation model in explaining why Aire-deficient mTECs show impaired *insulin 2* gene expression; the maturation model does not require the transcriptional hierarchies driving the development of authentic tissues. Instead, the maturation model has its own mTEC developmental process in which expression of particular TRAs is acquired at specific time points during the differentiation program. For example, expression of Aire-dependent TRA genes, such as *insulin 2* and *SAP1*, can be accomplished in terminally differentiated mTECs that have fully matured with the help of Aire protein. Lack of Aire in mTECs results in premature termination of differentiation, although Aire-deficient mTECs can still develop and pass a certain maturation stage. These Aire-less mTECs, which are rather mature (CD80^{high}) but not fully competent for TRA expression, have a more globular cell shape and lack transcriptional machinery and/or activity for Aire-dependent TRA genes (9) (**Figure 1**). In this scenario, Aire-independent TRA genes, such as *CRP* and *GAD67*, can be normally expressed even in Aire-deficient mTECs, because these TRAs can be expressed before the terminal differentiation stage(s), and consequently the lack of Aire has little impact on their expression. It is still unknown why some (Aire-independent) TRAs are expressed from the immature stage(s), whereas (Aire-dependent) others are expressed only after they become fully mature. For this reason, it would be important to clarify the exact timing of Aire expression during the course of mTEC differentiation (21). Nevertheless, promiscuous gene expression seems to be accomplished in terminally differentiated mTECs that have matured in the presence of Aire protein (the maturation model). Alternatively, reduced TRA gene expression could represent failure of heterogeneity in terms of TRA gene expression due to a halt in differentiation at a premature stage before heterogeneity of individual mTECs has occurred. In contrast, the transcription model may explain why some TRA genes are Aire-dependent and others are not, as outlined in the following. Aire-PHD1 binding with H3K4me0 is an interesting finding, and a model has been proposed suggesting that Aire's

PHD1 acts as a chromatin reader, searching for genes showing low expression (5, 22); Aire preferentially binds with weakly expressed genes harboring the silent chromatin signature of H3K4me0, and may help to up-regulate TRA genes whose expression levels would otherwise remain low (23). In this model, TRAs showing relatively high expression would do not require the help of Aire, and their expression would be Aire-independent.

One caveat of the transcription model is that expression of Aire and Aire-dependent genes does not always overlap at the single-cell level (19, 24, 25). If Aire were controlling the expression of TRAs in any way by means of direct transcriptional control, we would expect to see both Aire and Aire-dependent TRAs within the same individual cell, although one could argue that the timing of expression of Aire and Aire-target genes might not always be the same within any given analytical snapshot time frame.

Involucrin is an interesting TRA in that it is sometimes used as an example of an Aire-dependent TRA that follows the transcription model: when cultured cell lines were introduced with an Aire-expressing plasmid, transcription of the endogenous *involucrin* gene was up-regulated possibly due to the transcriptional activity of Aire (26). However, at the same time, involucrin is also used as a marker of mTEC maturation status. Similarly to its expression in the epidermis of the skin, involucrin is expressed by mature epithelial cells in the thymic medulla; it is expressed most strongly in Hassall's corpuscles, which seem to be the product of terminally differentiated mTECs, and Aire-deficient thymi show reduced numbers of involucrin-expressing mTECs, even in Hassall's corpuscles (9, 10). These findings are consistent with data favoring the maturation model derived from *in vivo* systems suggesting that Aire is required for promotion of the mTEC maturation program. Thus, there is a need to understand the types of effects that can be expected according to the experimental systems employed.

Regarding the role of Aire in the mTEC maturation program, an important issue that needs to be carefully investigated is whether or not Aire has proapoptotic activity. Introduction of Aire into cultured cells has been reported to result in apoptosis (11). Accordingly, increased MHC class II^{high}/$CD80^{high}$ mTECs seen in Aire-deficient mice was considered to explain the lack of Aire-mediated proapoptotic activity, because loss of Aire did not result in augmented proliferation of mTECs (11). Given that Aire plays an important role in the induction of a wide variety of TRAs, concomitant induction of apoptosis in Aire-expressing mTECs by Aire itself might be an effective way to promote cross-presentation, thereby facilitating negative selection (1). Once again, however, this attractive hypothesis needs to be investigated in more depth using *in vivo* experimental systems.

Finally, there is a need to discuss the implications of these two different models for the mechanisms underlying the defect of negative selection in Aire-deficient animals. In principle, the transcription model restricts the failure of negative selection in Aire-deficient mice to reduced expression of TRA gene products. In contrast, the maturation model suggests that Aire may affect the thymic microenvironment more globally than through simple control of TRA expression levels. Consequently, the maturation model allows for the possibility that regulation of TRA gene expression may not be the major defect of Aire-deficient mTECs responsible for impaired negative selection. Instead, other alterations in the function of mTECs lacking Aire might equally account for the defective negative selection in Aire-deficient mice. These changes could include processing and/or presentation of self-antigens within the mTECs (27), the process of thymocyte maturation (28), the process by which mature thymocytes are attracted to their proper location for negative selection by production of chemokines from mTECs (27, 29), control of cross-presentation through alteration of the relationship between BM-APCs and mTECs (30), and the balance between negative selection and regulatory T cell production (31). Furthermore, modification of microRNA-regulated TRA gene expression by Aire might represent another dimension in this field that warrants further investigation (32). All of the above issues may be largely clarified once the target genes of Aire have been determined using *in vivo* models. Thus, our current understanding of the fundamental function of Aire still seems to be in its infancy, and the proposal and evaluation of different models would doubtless lead to further advances in this fascinating field of research.

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Regulations of gene expression in medullary thymic epithelial cells required for preventing the onset of autoimmune diseases

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Elimination of potential self-reactive T cells in the thymus is crucial for preventing the onset of autoimmune diseases. Epithelial cell subsets localized in thymic medulla [medullary thymic epithelial cells (mTECs)] contribute to this process by supplying a wide range of self-antigens that are otherwise expressed in a tissue-specific manner (TSAs). Expression of some TSAs in mTECs is controlled by the autoimmune regulator (AIRE) protein, of which dysfunctional mutations are the causative factor of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). In addition to the elimination of self-reactive T cells, recent studies indicated roles of mTECs in the development of Foxp3-positive regulatory T cells, which suppress autoimmunity and excess immune reactions in peripheral tissues. The TNF family cytokines, RANK ligand, CD40 ligand, and lymphotoxin were found to promote the differentiation of AIRE- and TSA-expressing mTECs. Furthermore, activation of NF-κB is essential for mTEC differentiation. In this mini-review, we focus on molecular mechanisms that regulate induction of AIRE and TSA expression and discuss possible contributions of these mechanisms to prevent the onset of autoimmune diseases.

Keywords: medullary thymic epithelial cells, autoimmune disease, NF-κB, TNF receptor family, gene expression

INTRODUCTION

The thymus contributes to self-tolerance of T cells by eliminating potentially self-reactive T cells and generating immunosuppressive T cells, which are essential for preventing the onset of autoimmune disease. Epithelial cells localized in the thymic medulla [medullary thymic epithelial cells (mTECs)] are non-hematopoietic in origin and play non-redundant roles in the elimination of self-reactive T cells (1–4). Recent studies have revealed that mTECs also contribute to the selection and survival of immunosuppressive Foxp3-positive regulatory T cells (Tregs) (5–8).

Medullary thymic epithelial cells express several functional molecules required for the selection of self-tolerant T cells and Tregs (3). Mature types of mTECs express MHC molecules and co-stimulatory molecules essential for antigen presentation to developing T cells. In addition, mTECs secrete several types of chemokines (e.g., CCL19, CCL21, and CCL22) that attract T cells or dendritic cells in the medulla (2, 9). Moreover, a recent study has shown that the expression of CD70 in mTECs enhances the development and survival of Tregs via an interaction with its receptor, CD27, which is expressed on thymic T cells (5). A key feature of mTECs is their ability to express hundreds of self-antigens that are normally expressed in a tissue-specific manner (TSAs) (4, 10). TSAs are processed and directly presented by mTECs or indirectly presented by thymic DCs receiving TSAs from mTECs (4, 7, 11–13). T cells that recognize TSAs with high avidity undergo apoptosis (so-called negative selection) or survive as regulatory T cells (4, 14). Many studies have suggested significant roles of mTEC-dependent self-tolerance in preventing the onset of some autoimmune diseases in humans. Expression of some TSAs requires a nuclear protein autoimmune regulator (AIRE), the dysfunctional mutations of which are responsible for an inherited human autoimmune disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (15, 16). Whereas the expression of AIRE mRNA is detected in different cell types, AIRE expression at the protein level is remarkably high in mTECs (17). A previous study using AIRE-deficient mice provided evidence that autoimmunity, provoked by dysfunction of AIRE, is thymic stroma-dependent (18). In addition to APECED, recent studies have demonstrated that single-nucleotide polymorphisms (SNPs) in the *AIRE* gene are associated with rheumatoid arthritis (19, 20). In addition to mutations in the *AIRE* gene, reduced expression of the muscle acetyl choline receptor (*CHRNA1*) in mTECs was shown to be associated with the onset of myasthenia gravis (21). Moreover, impairment of the mTEC-dependent tolerance might explain the relationship between myocarditis and autoimmunity (22). These findings also imply that the onsets of various human autoimmune diseases could be related to dysregulation of mTEC-dependent tolerance. Interestingly, in addition to relationships with autoimmune diseases, recent studies have uncovered roles for mTEC-dependent T-cell tolerance in tumor tolerance (8, 23, 24).

Because expression of AIRE and TSAs is characteristic of mTEC, mTECs should harbor specific mechanisms to direct AIRE and TSA expression. Expression of TSAs appears to be correlated

with the differentiation of mTECs. In this mini-review, we specially focus on molecular mechanisms regulating the expression of AIRE and TSAs and the process of mTEC differentiation.

DEVELOPMENT OF mTECs

Thymic epithelial cells are classified into mTECs and cortical thymic epithelial cells (cTECs) (2). Several lines of evidence indicate the existence of a bi-potent TEC progenitor capable of differentiating into mTECs and cTECs in the fetal and adult thymus (25–29). The bi-potent TEC progenitor seems to give rise to each progenitor of mTECs and cTECs in the next stage (30, 31). Recent studies revealed that mTECs differentiate from progenitors expressing cTEC-markers (32, 33). These data imply that mechanisms determining the mTEC commitment suppress the cTEC-driving program. However, master molecules that decide the fate of the bi-potent TEC progenitor expressing cTEC-markers to the mTEC lineage have not been determined yet.

Currently, mTECs are classified based on the expression of MHC II, CD80, AIRE, and involucrin (**Figure 1**). mTECs (typically defined as $CD45^{-}$ $EpCAM^{+}$ $Ly51^{-}$ and $UEA\text{-}1^{+}$ by flow cytometric analysis) in adult mice are divided into two subpopulations, according to the expression levels of MHC II and CD80 (34). mTECs expressing high levels of MHC II and CD80 ($mTEC^{hi}$) express a more diverse set of TSAs than mTECs expressing lower levels of MHC II and CD80 ($mTEC^{lo}$) do (35). Moreover, precursor-product relationship analysis has suggested that the $mTEC^{lo}$ fraction can differentiate into $mTEC^{hi}$ (36, 37). Therefore, the $mTEC^{hi}$ fraction would be the more mature type of mTEC than $mTEC^{lo}$.

The $mTEC^{hi}$ fraction is further separated on the basis of AIRE expression (36, 38). Because previous studies have indicated that the AIRE-expressing $mTECs^{hi}$ ($AIRE^{+}$ $mTEC^{hi}$) are postmitotic and susceptible to apoptosis (36), $AIRE^{+}$ $mTECs^{hi}$ are postulated to be the more differentiated cell types than AIRE-negative $mTECs^{hi}$. mTECs expressing involucrin, a marker of terminally differentiated keratinocytes, are considered to be terminally differentiated mTECs that may be derived from $AIRE^{+}mTEC^{hi}$ (39, 40).

REGULATION OF AIRE mRNA EXPRESSION

Molecular mechanisms regulating the expression of AIRE, which are likely critical for preventing autoimmunity, remain unclear. In the fetal thymus, expression of AIRE starts at embryonic day 14.5 (41). Consistently, mature mTECs emerge around this embryonic day (42). Thus, AIRE expression seems to be closely linked to mTEC differentiation. However, because $mTEC^{hi}$ is separated into $AIRE^{+}$ and $AIRE^{-}$ fractions, the mTEC differentiation mechanism might be necessary but is not entirely sufficient for AIRE expression.

A study using a luciferase reporter assay identified a plausible minimal promoter region of the *AIRE* gene (43). This region contains binding sequences for Sp1, AP-1, NF-Y, and ETS family of transcription factors. Indeed, luciferase reporter analysis suggested regulation of the *AIRE* gene promoter by ETS family proteins (44). However, *in vivo* genetic studies are necessary to prove that these sequence-specific transcription factors are critical for the regulation of AIRE expression.

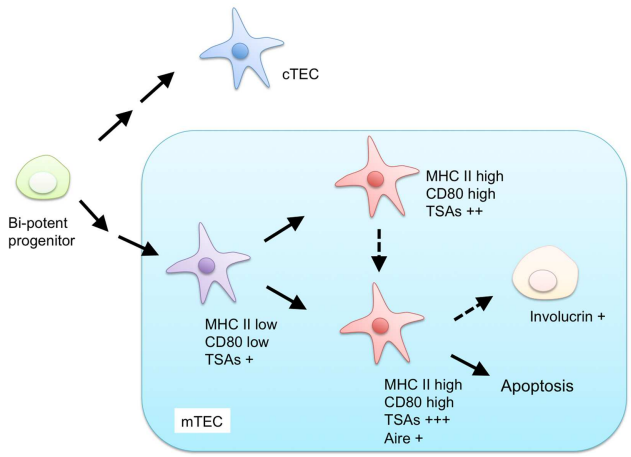

FIGURE 1 | Proposed model for differentiation of mTECs. Both mTECs and cTECs are generated from a bi-potent progenitor in the fetal and adult thymus. mTECs are classified by expression of MHC class II (MHC II), CD80, AIRE, and involucrin. mTECs expressing low levels of MHC II and CD80 are considered immature and give rise to mature mTECs, expressing high levels of MHC II and CD80, and a more diverse set of tissue-specific antigens (TSAs). MHC II-high and CD80-high mature mTECs are further separated into AIRE-positive and AIRE-negative subpopulations. AIRE-positive mature mTECs are postmitotic and undergo apoptosis or otherwise differentiate into involucrin-positive mTECs.

The promoter region of AIRE contains a high ratio of CpG sites (43). These CpG sites are hypermethylated in established cell lines defective in the AIRE expression. A subsequent study showed that these CpG sites are hypomethylated in isolated mTECs compared to thymocytes (45). These findings suggest that DNA demethylation might be prerequisite for AIRE expression. However, interestingly, hypomethylation was also observed in cTECs and thymoma with defective AIRE expression (45). Hence, DNA hypomethylation appears to be required but not sufficient for inducing AIRE expression.

Overall, AIRE expression seems to be regulated by combinations of chromatin modification and sequence-specific transcription factors. However, precise mechanisms and regulatory molecules remain to be determined.

REGULATION OF TSA mRNA EXPRESSION

TSA expression appears to be regulated by complicated mechanisms. Single-cell PCR analyses revealed a stochastic nature of TSA expression in mTECs (38, 46). Each TSA is expressed in a subset of mTECs (38, 46). The frequency of mTECs expressing a particular TSA was different, depending on the TSA (38, 46). Interestingly, various combinations of TSAs are expressed in individual mTECs (38, 46). These studies suggest that regulatory mechanisms of TSA expression in mTECs are different from those used in inherent tissues.

Several studies suggest that TSA expressions are epigenetically controlled. A comprehensive mRNA expression study revealed that TSA gene loci tend to co-localize in chromosomal clusters (35, 47). Moreover, genomic imprinting of the *Igf2* gene, a TSA, was lost in mTECs (35), implicating the involvement of

a DNA demethylation mechanism in TSA expression. Interestingly, another imprinted gene, *Cdkn1c*, was not affected. These data imply the existence of mTEC-specific mechanisms for demethylation of DNA.

Control of TSA gene expression by AIRE has been intensively studied (48–50). Several studies have revealed a function of AIRE as a transcription factor that directly promotes TSA expression (51, 52). Furthermore, AIRE binds to hypomethylated Histone 3 Lys 4 (H3K4) through its plant homology domain (53, 54). This finding suggests that AIRE modifies the chromatin structure in the TSA genes. AIRE also binds to DNA-PK (55–57), which functions in the repair of DNA-double strand breakage. A study using an mTEC cell line suggested that interactions of AIRE with H3K4 and DNA-PK are critical in recruiting AIRE to TSA gene loci and promoting TSA expression (57). Additionally, it was reported that AIRE interacts with P-TEFb, a component of the super elongation complex (58). It is generally accepted that transcription elongation, via the release of "paused" RNA polymerase II, is critical for the regulation of many genes (58, 59). AIRE may recruit P-TEFb to the TSA gene locus and promote elongation of the arrested TSA transcripts by releasing RNA polymerase II from the proximal promoter (60). Recent comprehensive analysis of mRNA transcripts in mTECs supports this mechanism (61). In addition to the TSA expression, the AIRE-dependent expression of some microRNAs (miRNAs) was recently revealed (62, 63). Consistently, genetic studies revealed important roles played by miRNA expressions in functions and maintenance of mTECs (63–65).

Compared to the mechanisms underlying Aire-dependent TSA expression, molecular mechanisms underlying Aire-independent TSA expression are less understood. As described above, whereas epigenetic regulations of TSA genes would be critical, mechanisms underlying epigenetic changes specific for mature mTECs remain unclear. Moreover, unidentified transcription factors may be involved in the promotion of Aire-independent TSA expressions.

EXTRACELLULAR SIGNALING TO PROMOTE DIFFERENTIATION OF mTECs EXPRESSING AIRE AND TSAs

Differentiation of TECs is well known to be correlated to differentiation of T cells in the thymus (so-called thymic cross-talk) (3). mTEC maturation was reported to be abolished in severe combined immunodeficiency (SCID) patients (66). This finding supports the idea that failure of the thymic cross-talk results in the onset of autoimmune manifestation through inhibition of mTEC function. Interestingly, a recent study showed that administration of anti-CD3ε antibody ameliorated autoimmunity in leaky SCID model mice possibly through improvement of the thymic cross-talk (67).

Molecular basis of the thymic cross-talk in mTEC development has been reported. Several lines of evidence revealed that TNF family cytokines expressed in thymocytes and other cells of hematopoietic origin (2) and their receptors expressed in mTEC are critical for the thymic cross-talk. Briefly, signaling of TNF receptor family members, RANK, CD40, and lymphotoxin-β receptor (LtβR), play essential roles in the development of mTECs expressing Aire and TSAs. This topic has been summarized in a recent review (1).

DOWNSTREAM OF TNF RECEPTOR FAMILY SIGNALING

TNF receptor family signaling induces the activation of NF-κB and MAPK pathways (68). To date, the involvement of the MAPK pathway in the development of mTEC remains to be addressed. However, several lines of evidence have indicated that the NF-κB family plays a critical role in the development of mTECs expressing AIRE and TSAs.

NF-κB members are sequestered in the cytoplasm in an inactive state by the binding of the inhibitory protein IκB in resting cells (69–71). Ligations of receptors induce phosphorylation and subsequent degradation of IκB proteins, thereby leading to nuclear localization of NF-κB to activate transcription. Two distinct NF-κB activation pathways, the classical pathway and the non-classical pathway, are currently known (70–72) (**Figure 2**). The classical pathway is required in inflammatory responses and lymphocyte activation (71). On the other hand, the non-classical pathway mainly promotes development and architecture formation of lymphoid organs, including the thymus. In the non-classical pathway, receptor ligation induces accumulation of the NF-κB-inducing kinase (NIK), which is normally degraded by the ubiquitin-dependent proteasome in resting cells. Subsequently, accumulated NIK phosphorylates and activates IKKα, which induces partial degradation of p100 to p52. p100 preferentially binds to and sequesters RelB in the cytoplasm, and the partial degradation of p100 to p52 induces translocation of RelB and p52 as a heterodimer into the nucleus.

The requirement for NF-κB activation in the development of mTEC was initially identified by the analysis of RelB-deficient mice (73, 74). RelB-deficient mice showed severe reduction in medulla size, accompanied by a lack of UEA-1-positive mTECs. Consistently, the expression of AIRE was abolished in the RelB-deficient thymus (6, 41, 75). As expected, RelB-deficient mice showed severe autoimmune diseases. A recent study demonstrated that autoimmunity of RelB mice was due to the defect in thymic stroma function (6). Mice carrying a dysfunctional mutation, NIK (*aly/aly*), also showed a similar defect in mTEC development and autoimmune phenotypes (76–78). Whereas IKKα-deficient mice die shortly after birth, neonatal IKKα-deficient mice and transplantation of IKKα-deficient thymic stroma indicates a requirement of IKKα in the development of mTECs (79, 80). mTEC development in p100-deficient mice is partially defective (81, 82), but this appears to be due to a partial rescue of p100 function by p105 (or its processed product, p50) because the double deficiencies of p100 and p105 resulted in severe defects in mTEC development, similar to the RelB- and NIK-mutant mice (83). Overall, these results support the idea that activation of the non-classical NF-κB pathway is essential for the development of mTECs.

TRAF6 is a signal transducer that mediates signaling from TNF receptor family members (84, 85). TRAF6-deficient mice exhibit severe autoimmune disease (86, 87). Additionally, recent studies suggest possible associations between SNPs of the *TRAF6* gene with rheumatoid arthritis and systemic lupus erythematosus in humans (88, 89). Previous studies showed that TRAF6 promotes the development of mTECs expressing AIRE and TSAs, thereby suppressing autoimmunity (86). Moreover, RANK-mediated differentiation of mTECs requires TRAF6 in *in vitro* organ culture

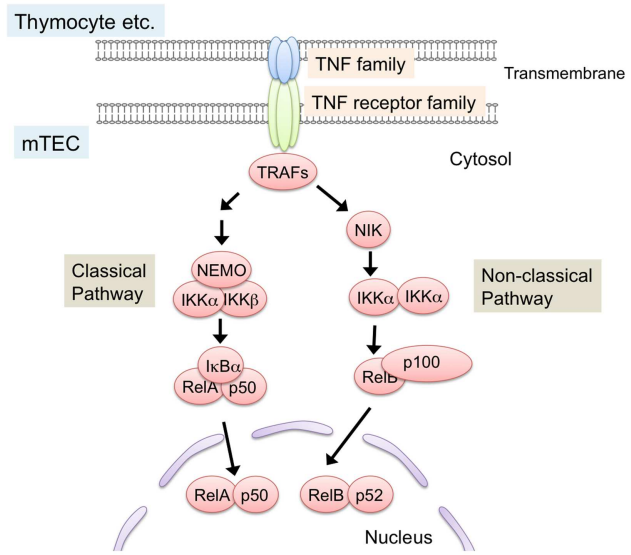

FIGURE 2 | NF-κB activation pathways triggered by TNF family signaling. Interaction of TNF family ligand (RANK ligand, CD40 ligand, and lymphotoxin α and β complex) with their respective receptors (RANK, CD40, and LtβR) induces activation of NF-κB pathways. Interaction between the ligand and its receptor induce the binding of TRAF-family proteins to the cytoplasmic domains of TNF receptors. TRAF-family proteins in turn activate downstream serine/threonine kinase cascade. These kinases trigger the degradation of inhibitory proteins that sequester NF-κB in cytosol, thereby leading to the translocation and transcriptional activation of NF-κB members. NF-κB pathways are classified into classical and non-classical pathway. In the non-classical pathway, NF-κB complex consisting of RelB and p52 is activated. NIK is critical for the non-classical NF-κB pathway. TRAF6, a member of the TRAF protein family, was reported to regulate only the classical NF-κB pathway, which causes nuclear translocation of mainly the RelA complex. On the other hand, other TRAF members function in the non-classical NF-κB pathway by binding to the TNF family receptors.

of fetal thymic stroma (90). Notably, TRAF6 is a signal transducer that mediates the activation of the classical NF-κB pathway but not the non-classical NF-κB pathway (84, 85). Thus, these data imply a role for TRAF6-mediated activation of the classical NF-κB pathway in mTEC differentiation.

In addition to the above findings, a scaffold protein, Sin (also called Efs), was proposed to be expressed downstream of TNF receptor family signaling. Sin-deficient mice showed reduced numbers of mTECs and thymic stroma-dependent autoimmunity (91). In addition to the role of Sin in FGF-mediated proliferation signaling (91), a recent study suggested that Sin might regulate the non-classical NF-κB pathway activated by RANKL signaling (92). Because the SH3 domain and phosphorylation of tyrosine residues of Sin might be critical for its function (93, 94), these studies also imply unrecognized roles of Src-type tyrosine kinases in mTEC development.

CONCLUDING REMARKS

Whereas significant roles for NF-κB in signal activation of mTEC differentiation and subsequent expression of AIRE and TSAs are indisputable, molecular events connecting these signaling pathways to induction of AIRE and TSA remain unclear. It was reported that LtβR signaling induces the expression of AIRE in an mTEC line in the presence of a DNA methylation inhibitor (95). However, it is still unclear whether NF-κB binds to the promoter of the *AIRE* gene. Moreover, a wide variety of TSA expression would not be explained only by NF-κB-dependent transcriptional activation because NF-κB family members are generally known to be sequence-specific transcription factors. Thus, the link between NF-κB activation and expression of AIRE and TSAs remains largely enigmatic.

In addition, differentiation stages regulated by these signaling molecules and their mechanisms need to be clarified. mTECs have different properties in each developmental stage, with regard to TSA expression, AIRE expression, and DNA methylation status. Therefore, it is important to clarify types of mTECs in which each TNF receptor family signal functions. Overall, more studies are needed to understand the molecular and cellular mechanisms regulating the development of mTECs with the final aim to develop novel therapeutic strategies preventing autoimmune diseases caused by defective thymic functions.

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Human APECED; a sick thymus syndrome?

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Loss-of-function mutations in the Autoimmune Regulator (AIRE) gene cause a rare inherited form of autoimmune disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, also known as autoimmune polyglandular syndrome type 1. The patients suffer from multiple endocrine deficiencies, the most common manifestations being hypoparathyroidism, Addison's disease, hypogonadism, and secondary amenorrhea, usually accompanied by typical autoantibodies against the target tissues. Chronic mucocutaneous candidiasis is also a prominent part of the disease. The highest expression of AIRE is found in medullary thymic epithelial cells (mTECs). Murine studies suggest that it promotes ectopic transcription of self antigens in mTECs and is thus important for negative selection. However, failed negative selection alone is not enough to explain key findings in human patients, necessitating the search for alternative or additional pathogenetic mechanisms. A striking feature of the human AIRE-deficient phenotype is that all patients develop high titers of neutralizing autoantibodies against type I interferons, which have been shown to downregulate the expression of interferon-controlled genes. These autoantibodies often precede clinical symptoms and other autoantibodies, suggesting that they are a reflection of the pathogenetic process. Other cytokines are targeted as well, notably those produced by Th17 cells; these autoantibodies have been linked to the defect in anti-candida defenses. A defect in regulatory T cells has also been reported in several studies and seems to affect already the recent thymic emigrant population. Taken together, these findings in human patients point to a widespread disruption of T cell development and regulation, which is likely to have its origins in an abnormal thymic milieu. The absence of functional AIRE in peripheral lymphoid tissues may also contribute to the pathogenesis of the disease.

Keywords: APECED, AIRE, T cells, autoimmunity, thymus

INTRODUCTION

Monogenic diseases, although rare, provide a unique possibility to obtain information in the human system on the significance and function of the molecules affected by the mutations. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also known as autoimmune polyendocrine syndrome type 1 (APS-1), is one such natural knockout phenotype, and has provided important information on T cell selection and pathogenesis of organ-specific autoimmunity (1, 2). It is a recessively inherited human autoimmune disease, caused by loss-of-function mutations in the Autoimmune Regulator (AIRE) gene (3, 4). It is enriched in certain populations, most notably the Finns (prevalence 1:25 000), Sardinians (1:14 000), and Iranian Jews (1:9 000) (5). The pathognomonic triad consists of chronic candidiasis, hypoparathyroidism, and Addison's disease, with several other endocrine and non-endocrine manifestations affecting a smaller fraction of the patients.

Abbreviations: AIRE, autoimmune regulator; ALAT, alanine amino transferase; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; APS-1, autoimmune polyendocrine syndrome type 1; CMC, chronic mucocutaneous candidiasis; GAD, glutamic acid decarboxylase; LKM, liver-kidney microsomal; mTEC, medullary thymic epithelial cells; RTE, recent thymic emigrant; TRA, tissue-restricted antigen; Treg, regulatory T cell.

Since the discovery of the underlying genetic defect in 1997, the pathogenesis of this rare polyendocrine syndrome has been the focus of considerable interest. In particular, studies in Aire-deficient mice have shown that AIRE plays an important role in T cell development and negative selection in the thymus, thus elucidating general pathways of thymic development (2). However, the murine phenotype differs in several key points from the human disease, not least in the absence of all the defining triad components mentioned above (6). It is clear that to understand how AIRE works in the human immune system, the human disease mechanisms have to be studied on their own terms, and not only as an extension of the murine phenotype. Such an approach has already proved successful, for example by revealing the role of anti-cytokine antibodies in causing the increased susceptibility to *Candida* infections (7). Here, we review the main features of human APECED, both clinical and immunological. Although several important questions remain open, we also attempt to provide an explanation of the pathogenetic mechanisms, looked at from the human viewpoint.

CLINICAL FEATURES

The classic triad of APECED consists of Addison's disease, hypoparathyroidism, and chronic mucocutaneous candidiasis

(CMC), two of which are required for the diagnosis. The autoimmunity in APECED is organ-specific but with many components. There is no gender bias. Virtually all patients have more than two disease components and up to 10 components have been reported. On the average patients have four components (8). First symptoms occur on average at the age of five (range 0.2–18 years) (9). The most common endocrinopathies, hypoparathyroidism, and Addison's disease are usually diagnosed at the age of 3–5 years and 11–15 years, respectively. New components appear through life (9).

Chronic mucocutaneous candidiasis is usually the first sign of APECED and in Finnish patients its prevalence is 100%. Candidiasis is the most common component of APECED except in Iranian Jews in whom it is rarely described (10). The severity of the symptoms varies from redness and soreness of the corners of the mouth to inflamed mucosal surfaces in the whole oral cavity. Candidiasis increases the risk of oral carcinoma by causing chronic inflammation (1).

Hypoparathyroidism is the most common autoimmune component in APECED and APECED should be considered in the differential diagnosis of every patient with hypoparathyroidism of unknown cause. Addison's disease is the second most common autoimmune component and its prevalence was 78% in a large patient series (9). It most often presents with both mineralo- and glucocorticoid deficiency. Gonadal failure is a common component, especially in women. Ovarian insufficiency is actually the third most common autoimmune component, affecting approximately 65% of APECED women and starting in early adulthood (9). It can present with primary amenorrhea with a complete failure of or arrested pubertal development. About half of the cases develop premature menopause (9). Testicular failure has a maximum prevalence of 25% in men and starts usually at an older age (11).

About one third of APECED patients develop hypothyroidism, usually after puberty (9). Thyroid autoantibodies are found commonly but clinical disease is not always present. The prevalence of type I diabetes mellitus among APECED patients varies between populations. In a large Finnish patient series, the prevalence was about 30% (8). Gastrointestinal symptoms, such as chronic diarrhea, constipation, hepatitis, and gastritis are common. Autoimmune gastritis with pernicious anemia is present in approximately 30% of patients by middle age (9). In severe forms, patients develop chronic atrophic gastritis and pernicious anemia (9). About 20% of patients develop autoimmune hepatitis with variable severity (8).

Ectodermal manifestations include enamel hypoplasia of the teeth, alopecia, nail dystrophies, vitiligo, and ocular keratopathy (8, 9). Keratitis occurs in 25% of the patients and can lead to vision loss. Keratitis can be an early and even the first manifestation of the disease. Alopecia and vitiligo affect up to 30–40% of the patients by middle age (5, 12).

DIAGNOSIS AND AUTOANTIBODY FINDINGS

The diagnosis of APECED is based on the clinical features, detection of autoantibodies, and genetic analysis. Two of the most common disease components are required for APECED diagnosis. Candidiasis is usually the first symptom, while hypoparathyroidism and Addison's disease are the most common endocrinopathies. Since the gene test is available, the diagnosis is confirmed with the identification of the mutation. The type of mutation, however, does not predict the disease course. Due to the rarity of APECED, there is often delay of years before the diagnosis is set (13).

An important feature in the diagnostics is the existence of IgG autoantibodies. Their possible pathogenetic role is mostly unknown. Many autoantibodies in APECED are targeted against intracellular enzymes (14). It is possible that the detected autoantibodies are not pathogenetic but, instead, are a marker of the ongoing T cell activity at the target tissue (14). The presence of autoantibodies correlates with the disease components but autoantibodies can also precede the onset of the target organ failure (15). Some autoantibodies are closely associated with the corresponding disease manifestation, while others are found only in a subset of patients with the particular manifestation. For example, autoantibodies against calcium-sensing receptor are found in almost all APECED patients with hypoparathyroidism, whereas anti-NALP5 antibodies, also linked to hypoparathyroidism, are much less common (16, 17). Also, autoantibodies found in APECED patients may be different from those found in patients with an isolated autoimmune disease. A case in point is GAD (glutamic acid decarboxylase), an important autoantigen in type I diabetes but rarely targeted in APECED patients with diabetes as a disease component (18).

Once the initial diagnosis is made, APECED patients must be evaluated regularly and tested for autoantibodies, since new disease components appear through life (19). Suggestions for laboratory testing in the follow-up of APECED patients are presented in **Table 1**.

The recently discovered neutralizing antibodies against type I interferons are found in 100% APECED patients (20, 21). Anti-interferon antibodies are present before symptom development at high titers. In addition to APECED, type I interferon autoantibodies have been found only in patients with thymoma, but with lower prevalence and titers (20, 21). Thus, measurement of neutralizing

Table 1 | Suggested tests in suspected APECED and for the follow-up of APECED patients.

Disease component	Autoantibody	Other tests
APECED	Interferon-α and/or interferon-ω	
Hyperparathyroidism	NALP5	Plasma calcium
	Calsium-sensing receptor	Plasma phosphate
Addison's disease	21-hydroxylase	Plasma renin
	Adrenocortical antibodies	Plasma ACTH
Diabetes mellitus type I	IA-2	
Hypothyroidism	Thyroid peroxidase	
Gonadal insufficiency	Steroid cell antibodies	FSH, LH, estrogen
Gastritis	Parietal cell antibodies	vitamin B12
Hepatitis	LKM antibodies	ALAT

In diagnosed APECED patients the aim is to screen for new disease components for early diagnosis.

antibodies against the type I interferons α and ω is a sensitive diagnostic test.

Chronic mucocutaneous candidiasis correlates with autoantibodies against Th17 class cytokines, both in APECED patients and CMC patients without APECED (22–24). The autoantibodies are neutralizing and have been found against IL-22 (91% of patients), IL-17F (75%), and IL-17A (41%) (22). Testing for these antibodies is not commonly used in the diagnostic evaluation, since the abovementioned type I interferon antibodies are more prevalent and specific.

NACHT leucine-rich-repeat protein 5 (NALP5) is a protein expressed in the cytoplasm of parathyroid chief cells. Forty-nine percent of APECED patients with hypoparathyroidism are positive for NALP5 antibodies (16). These antibodies are not found in APECED patients without hypoparathyroidism or in non-APECED patients with hypoparathyroidism. NALP5 antibodies thus represent APECED-specific autoantibodies.

Adrenocortical autoantibodies can be detected by indirect immunofluorescence assays. These antibodies recognize 21-hydroxylase, 17-hydroxylase, and side-chain cleavage enzymes, which are involved in the steroid hormone synthesis (11). Ovarian insufficiency correlates with the presence of autoantibodies against side-chain cleavage enzyme (11). Steroid cell autoantibodies can be screened for by indirect immunofluorescence assays with ovarian and testicular tissues. APECED patients with ovarian insufficiency also have elevated FSH and LH levels and low estrogen levels (1).

Fifty percent of APECED patients with hepatic involvement have liver-kidney microsomal (LKM) antibodies (25). The cytochrome target antigen is CYP1A2. Autoantibodies against CYP1A2 are highly specific (100%) but their sensitivity is only 50% (12). Plasma alanine amino transferase (ALAT) are a good marker for developing autoimmune hepatitis in APECED (1). Smooth muscle cell antibodies, which are a marker for autoimmune hepatitis, are not commonly present in APECED-associated hepatitis (25).

In APECED, autoantibodies against IA-2 (tyrosine phosphatase-like protein IA-2) correlate with the development of type I diabetes. However, the sensitivity is low (11). GAD antibodies are relatively common (33%) in APECED patients but their presence does not correlate with diabetes, in contrast to non-APECED patients (11, 18).

Gastrointestinal symptoms, for example constipation and diarrhea are common among APECED patients. Hypocalcemia due to hypoparathyroidism can cause diarrhea. Exocrine pancreatic failure occurs in a few percent of patients and can present with malabsorption and steatorrhea (9). Plasma calcium levels and exocrine pancreatic function should be assayed in APECED patients with diarrhea.

GENETICS OF APECED

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy is caused by loss-of-function mutations in the *AIRE* gene. The human *AIRE* gene is found in the q22 region of chromosome 22, and shares a 71% sequence homology with its murine counterpart Aire (3, 4, 26). At full length, AIRE encodes a 58 kDa protein of 545 amino acids, although two other, shorter splice variants of unknown significance have been described (4, 27). AIRE contains a N-terminal caspase-recruitment domain and SAND domain that together regulate AIRE's multimerization, two plant homeodomains (PHDs), and a proline-rich region (**Figure 1**) (28, 29). The N-terminus also contains a nuclear localization signal, while C-terminus is important for transcriptional activation. PHDs are zinc fingers involved in protein-protein interaction, and PHD1 has been shown to mediate the binding of AIRE to non-methylated histone H3 (30–34). Together, these sequence and structural features suggest a role in the regulation of gene transcription, but despite some data suggesting otherwise (35), it is unlikely that AIRE is a transcription factor that directly binds DNA.

Today, more than 60 APECED mutations have been identified. The mutations are found throughout the coding region in AIRE and include nonsense mutations causing premature stop codons, frameshifts caused by deletions and missense mutations (36). The most common ones are the mutations R257X (c.769C > T) in exon 6 and a 13-base pair deletion (c.967–979del) in exon 8 (37, 38). APECED is inherited in an autosomal recessive manner and heterozygosity does not cause APECED. However, there is one Italian family where a dominantly inherited mutation in the AIRE gene (G228W) causes APECED (39).

In general, the type of mutation in APECED does not correlate with the clinical features (40). Phenotype can vary even between siblings carrying the same mutation (9). However, the R257X mutation carriers have more commonly candidiasis than patients with other mutations (40). Also, Iranian Jews APECED patients have a unique mutation (Y85C, c.374A > G) and do not develop keratopathy or candidiasis (10).

AIRE's EXPRESSION AND FUNCTION

A few years after the discovery of the genetic defect responsible for APECED, experiments with knockout mouse models linked AIRE to the transcription of tissue-restricted antigens (TRAs) in thymus. The highest expression of AIRE is found in medullary thymic epithelial cells (mTECs) (41), which are capable of expressing a diverse set of genes normally restricted to certain tissues (42, 43). This phenomenon has been denoted ectopic transcription and is likely to be important for the deletion of autoreactive thymocytes. In the absence of Aire a subset of thymic TRAs was

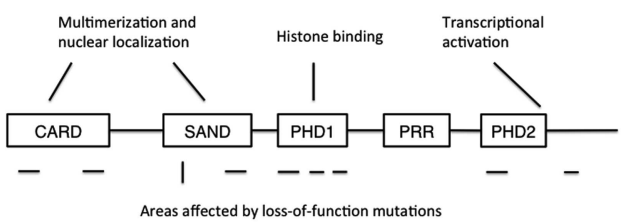

FIGURE 1 | The structure of AIRE protein. CARD: caspase recruitment domain; SAND: human Sp100, Aire1, NucP41/P75, and *Drosophila* DEARF1 domain; PHD: plant homeodomain; PRR: proline-rich region. CARD and SAND are associated with AIRE multimerization and nuclear localization, while PHD1 mediates binding to non-methylated histone H3. Mutations disrupting AIRE's function have been found in all domains except PRR, and also at the extreme C-terminus. Areas where recessive mutations have been found are shown below the schematic structure as horizontal lines, while the location of the dominant mutation is shown as a vertical line.

down-regulated, suggesting that Aire functioned as a regulator of the ectopic transcription of some TRAs in mTECs (44). Another study using a model antigen with Aire-regulated promoter provided evidence of increased development of autoreactive T cells and subsequent autoimmunity when Aire was knocked out, at least in that particular transgenic system (45). Later studies have confirmed but also complicated the link between TRA transcription and Aire (2).

It has been difficult to establish how AIRE facilitates TRA transcription, given the promiscuous nature of its function and the diverse and context-dependent set of genes affected by AIRE's absence. Several studies have reported that AIRE can bind DNA, suggesting that it might function as a transcription factor (35, 46). Others, however, have challenged the significance of direct DNA binding by AIRE (32, 47, 48). Moreover, the genes regulated by AIRE lack shared promoter elements (47), and it is thus difficult to see how AIRE could by direct DNA binding regulate such a diverse set of genes.

A detailed analysis of AIRE's molecular partners suggests a less direct role for AIRE in gene transcription. A common feature of AIRE-regulated genes is that they are associated with a stalled RNA polymerase II (RNAPII), which stops full-length transcription (34, 49, 50). AIRE then seems to allow further elongation of the target genes. A recent attempt to formulate a model suggests that AIRE first binds to non-methylated histone H3 (29). It then recruits positive transcription elongation factor b (P-TEFb) to phosphorylate RNAPII, reactivating the stalled polymerase and gene transcription. Although this would allow for a broad range of genes to be activated, a major problem with this model is to explain how AIRE's control on TRAs is maintained even when the TRAs are expressed as transgenes, outside of their normal epigenetic environment.

A competing explanation is that AIRE is not directly involved in TRA transcription, but rather regulates mTEC maturation and death, thus indirectly affecting also mTEC-expressed TRAs. In support of this possibility are numerous studies showing that Aire-deficiency results in fundamental changes in the mTEC population (51–54). The expression of AIRE is a late event in the lifespan of mTECs (55), and it has been suggested to be a terminal differentiation factor for mTECs. Thus, in the absence of AIRE, the altered developmental pathway of mTECs might lead to a decrease in the TRA-expressing stages, either because of abnormal cell death or diverted or arrested maturation. However, although the mTEC differentiation model is compatible with most reported effects of AIRE-deficiency, the molecular mechanisms by which AIRE regulates mTEC biology are largely unknown.

The strengths and weaknesses of scenarios involving regulation of gene transcription versus regulation of mTEC homeostasis have been more fully summarized in a recent review (47). At the moment the available data do not provide unequivocal support to either of the two main models, and it should also be noted that practically all the data come from studies on the murine system or cell lines. Nevertheless, we would argue that the human phenotype is difficult to reconcile with models invoking regulation of TRA transcription as the main function of AIRE. We discuss these aspects in more detail below, in the chapter on the pathogenesis of APECED.

A further complication is AIRE's expression in peripheral tissues, a phenomenon of largely unknown significance (56). Early studies showed AIRE mRNA expression in a wide range of tissues, but not all of these studies were confirmed on protein level. In the periphery AIRE is expressed at significantly lower levels than in the mTECs, making reliable detection with mostly polyclonal antibodies difficult, and some studies have questioned the presence of any extrathymic AIRE (57). Nevertheless, a wide agreement exists for the expression of AIRE in lymphoid tissues, with perhaps dendritic cells as the main AIRE+ population (58, 59). The peripheral AIRE+ cells can also express TRAs, although the genes are only partly overlapping with the thymic set (60).

IMMUNOLOGICAL ABNORMALITIES

The most obvious immunopathological finding in APECED patients is the diverse set of autoantibodies, mostly against tissues affected by the disease. The autoantibodies are often directed against enzymes involved in hormone synthesis, but since these enzymes are intracellular, the organ-specific autoantibodies rarely have pathogenetic significance. Of special interest are the neutralizing autoantibodies to cytokines, since they are likely to have pathogenetic effects. These autoantibodies are found in practically all APECED patients, while extremely rare in healthy people, and can block interferon-induced gene expression both *in vivo* and *in vitro* (21, 61). The more recently described neutralizing autoantibodies against the Th17 cytokines IL-17A, IL-17F, and IL-22 are linked to the defective antifungal defense and CMC (22, 24).

However, the organ-specific autoimmune manifestations of APECED are generally held to be T cell-mediated, and therefore the T cell compartment in APECED patients has been studied in a number of studies. A recent study showed that APECED patients have a significantly increased frequency of highly differentiated CD8+ effector T cells (62). These cells express CD45RA and lack CCR7, a phenotype similar to that found in some chronic viral infections, and express cytotoxic molecules, such as perforin. The specificity of these cells is not known, but it is likely that at least some of them represent the autoreactive population.

The CD4+ population likewise shows changes, but so far the data are scant and partly contradictory. An increased frequency of CD25 + CD4+ cells, including both regulatory and activated populations, was reported in one series of patients (63), while a later study failed to find differences between patients and controls in the frequency or number of CD4+ activated/memory T cells (64). With the discovery of anti-cytokine antibodies the cells producing Th17 cytokines have also been studied. Despite the presence of anti-IL17 antibodies, IL-17A production is generally normal or even increased, while IL-17F is reduced. An even greater reduction is found in IL-22 responses (22, 65, 66). These defects are closely linked to the chronic candidiasis.

The best-defined T cell defects, confirmed by several studies, are found in the regulatory T cell (Treg) population. The earliest report showed that APECED patients have a decrease of CD25 + CD4+ Tregs, a finding later confirmed in another cohort (64, 67). A more detailed examination was facilitated by the discovery of FOXP3 as the key transcription factor in Tregs, and subsequent development of mAb allowing the identification of FOXP3+ cells. APECED patients have a decreased frequency and number of FOXP3+ Tregs,

and in a single-cell analysis the Treg cells express reduced levels of FOXP3 protein (68). Moreover, in an *in vitro* co-culture assay Tregs isolated from APECED patients show defective suppressive function. The most clear-cut Treg abnormalities are found in the CD45RO+ activated subset, the population mostly responsible for the regulatory activity (62, 69). Cells of the innate immune system have also been analyzed, especially those in which AIRE is expressed. The frequency of circulating dendritic cells has been reported to be normal (64, 70), but several studies suggest functional changes, at least in monocyte-derived dendritic cells (58, 71, 72). However, here again the published results have been partly conflicting, with some reporting hyperactivation of dendritic cells, and others defective cytokine production and functional impairment. The expression of pattern-recognition receptors has also been studied and seems normal (70).

An altogether different putative role for AIRE links it with Dectin-1, a receptor of the innate immune system (73). AIRE was reported to form transient complexes with Dectin-1 pathway components and localize with Dectin-1 at the cell membrane. In APECED patients peripheral blood mononuclear cells the production of TNF-α in response to Dectin-1 ligation was reduced. Because Dectin-1 is important in innate recognition of β-glucan and anti-*Candida* responses, these findings offer an alternative mechanism for the defective antifungal defense in APECED patients.

With the exception of the increased susceptibility to *Candida*, the general view emerging from these studies, perhaps not unexpectedly, is one of increased effector activity and decreased regulation. As in all human studies, two major problems complicate the interpretation of these results. First, the analysis is restricted to circulating cells, which are likely to be at best a partial reflection of the local autoimmune process. Secondly, most of the studies have been performed on adult patients with long-established disease, and care is needed to separate primary pathogenetic factors from secondary effects of the disease process. We will discuss pathogenesis below. Nevertheless, it is also important to note that not all secondary processes are irrelevant to the pathogenesis. In most patients, new targets of autoimmunity and new disease manifestations continue to appear later in life, and it is likely that the general immune dysregulation is a contributing factor in this unpredictable progression of the disease.

PATHOGENESIS

Given the expression pattern of AIRE, it is highly likely that the thymus is in a central role in the pathogenesis of APECED. The simplest putative explanation for the disease is based on the link between AIRE and ectopic transcription of TRAs in the thymus. In this model the absence of AIRE-regulated TRAs in the thymus disrupts negative selection and allows the escape of autoreactive T cells to periphery, which leads to organ-specific autoimmunity (**Figure 2**). Support for this scenario comes from murine studies, and in particular transgenic settings with Aire-regulated model antigens (44, 45, 74, 75).

However, several aspects of both the murine and human AIRE-deficient phenotype are very difficult to reconcile with this simple model. A corollary of TRA-driven autoimmunity is that at least the earliest events should be specific to AIRE-regulated TRAs and

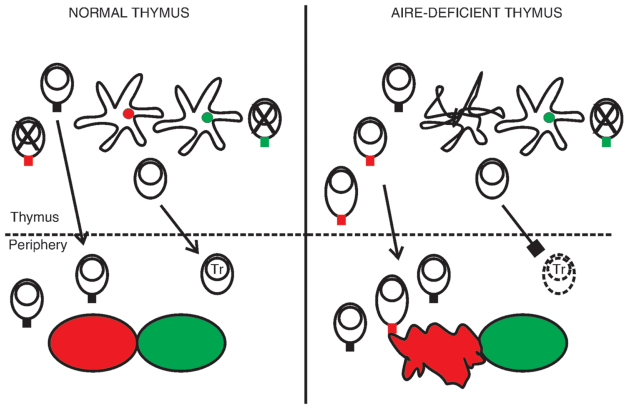

FIGURE 2 | A hypothesis of the pathogenesis of APECED. In the normal thymus mTECs express a range of tissue-restricted antigens, shown here as red and green circles within the mTECs. This facilitates the deletion of autoreactive thymocytes, shown as cells with a red or green TCR, so that only T cells reactive to non-self antigens migrate to the periphery. The normal thymus also supports the development of regulatory T cells (Tr). In the absence of functional AIRE the epithelial compartment is disrupted, as indicated by the malformed mTEC, and some of the TRAs are no longer transcribed. Thymocytes specific to these TRAs mature in increased numbers. They may also become activated already in the thymus, shown here as an increase in cytoplasm volume, so that the thymus exports functional effector cells. At the same time the development of Tr cells is defective, leading to insufficient suppression in the periphery. The outcome is organ-specific autoimmunity, at least partly directed by the range of TRAs missing in the AIRE-deficient thymus (here indicated by the red tissue).

the response limited to clones that escaped negative selection. In the murine system some of the reported manifestations involve antigens that are independent of AIRE, suggesting that loss of TRAs is not an obligatory prerequisite of the autoimmunity (76). In humans, perhaps the main problem for the TRA model is to explain the early and universal incidence of anti-interferon antibodies. As noted earlier, these antibodies can precede any clinical symptoms or organ-specific autoantibodies (5), strongly suggesting that their appearance is an important part of the pathogenetic process, or at least reflects it. It is very difficult to see how, without any additional defects, the loss of TRAs in mTECs alone could give rise to this particular phenomenon. Another difficulty arises from the study of thymomas. The disorganized thymoma tissue often lacks AIRE expression, yet continues to support T cell maturation. If the loss of TRAs and subsequent escape of autoreactive T cells would suffice to cause APECED, a substantial fraction of thymoma patients should develop it. Yet the patients rarely show APECED-like manifestations (77, 78). Instead, autoimmunity associated with thymomas is dominated by myasthenia gravis, which conversely is not a manifestation of APECED (9, 79).

An alternative model suggests that the loss of AIRE leads to a more extensive disruption of the thymic microenvironment, creating conditions that favor activation instead of tolerance. Although a primary lymphoid organ, thymus is also capable of developing tertiary lymphoid organization, with germinal centers and induction of adaptive immunity (5). The unique feature of thymus is that, unlike in the secondary lymphoid organs, many of the T cells

inhabiting the organ have not yet passed negative selection and are potentially highly autoreactive. The crucial difference when compared with the TRA model is that here the thymus would export preactivated autoreactive T cells, perhaps in high numbers, and not naive, clonally restricted autoreactive precursors. Moreover, since the absence of AIRE is also likely to disrupt the development of natural Tregs (80–82), the defect in immunoregulatory mechanisms contributes to the emerging autoimmunity.

Several observations support the view that a more widespread thymus disturbance than decreased TRA expression alone is responsible for APECED. First, thymoma patients also develop anti-interferon antibodies (83), a feature shared only by these two diseases affecting the thymus, and resected thymoma tissue often shows tertiary lymphoid structures (79, 84). Secondly, in APECED patients even cells expressing markers typical of naive lymphocytes, e.g., CD45RA and CCR7, show clear signs of functional activation, such as the expression of perforin (62). Interestingly, this also applies to CD8+ T cells expressing CD31, a marker of recent thymic emigrants (RTEs). This is consistent with activating events taking place already in the thymus, so that the cells migrate to the periphery preactivated. Similarly, the CD31+ subset among resting Tregs is clearly abnormal, suggesting that the Treg cell defect is also traceable to the thymic development (80). And thirdly, indirect support is provided by the studies showing disruption of thymic medulla in the absence of functional Aire. A dysregulated thymus functioning as an induction site for autoimmune responses would also explain the relatively early onset of the disease.

Nevertheless, the fact that most patients develop similar main components of the disease suggests that the initiation of autoimmunity does show predilection to certain self antigens. It is therefore likely that the range of TRAs missing from the AIRE-deficient thymus, whether primarily due to transcriptional failure or disrupted mTEC development, defines at least to some extent which peripheral organs are targeted. The early pathogenetic events in the thymus would thus be a combination of a general failure to imprint tolerance and a clonally restricted targeting of a subset of potential self antigens.

This thymus-centered view on the pathogenesis of APECED leaves important questions open, including the significance of peripheral AIRE. So far, this is issue is largely unknown, apart from what can be extrapolated from murine studies and the observed changes in the characteristics of dendritic cells and other AIRE+ peripheral cells when AIRE is absent. Likewise, AIRE's interaction with Dectin-1 partners and its contribution to the antifungal defense remains to be defined.

Moreover, the disease manifestations traditionally held to be caused by factors other than autoimmunity need to be re-examined. The recent data on neutralizing antibodies against Th17 cytokines, and on the importance of these cytokines in anti-Candida defense strongly suggests that the chronic candidiasis is basically an autoimmune phenomenon, too (7). It may also be questioned whether the ectodermal disease components represent developmental dystrophies, as was originally believed. The argument against this view holds that since they are not congenital but develop later, a primary defect is unlikely. However, with the exception of such clearly autoimmune manifestations as alopecia and vitiligo, the pathogenesis of ectodermal components is still unknown.

AIRE IN OTHER DISEASES

Because the effects of AIRE-deficiency are so drastic, many studies have addressed the possibility that heterozygous mutations or genetic variants of AIRE might also predispose to autoimmunity. So far, the results are intriguing but inconclusive and to some extent contradictory. Studies on the first-degree relatives of APECED patients have generally failed to find a link between heterozygous carriage of AIRE mutations and autoimmune diseases (85, 86), although some data suggesting otherwise have been reported (87). A clear limitation in all such studies is the small number of study subjects. Another approach has been to search for AIRE mutations in patients with isolated autoimmune diseases. In most cases heterozygotes have not been found to be enriched among the patients (88, 89), but again there are some conflicting data (90). However, several recent studies suggest that single-nucleotide polymorphisms in the AIRE gene are associated with an increased risk of autoimmunity, including rheumatoid arthritis and vitiligo (91–93). It is therefore probable that more detailed analysis of AIRE will reveal more instances in which genetic variation in AIRE, presumably leading to modulation of its function, affects the predisposition to non-APECED autoimmunity.

CONCLUDING REMARKS

Despite the simple genetics of AIRE, the resulting phenotype is highly complex, and the disease manifestations can vary greatly between patients with identical mutations. The significance of this complexity has sometimes been dismissed by attributing it to secondary effects of a longstanding disease or the genetic heterogeneity of the patients. Although both arguments are relevant, they can also be a too facile way to sidestep important issues. The simple model of reduced TRA expression as the main mechanism of APECED is increasingly untenable, so alternative and additional mechanisms must be considered, and human patients studied to test them. Moreover, although the genetic diversity of the human patients certainly influences and complicates matters, it must be accepted and addressed. The relative simplicity of inbred Aire-deficient animal models is attractive but also potentially deceptive. After all, in the end the results have to be taken back to human patients, when the outbred nature of the subjects is an unavoidable fact.

The existing data indicate that the earliest pathogenetic events leading to APECED take place in the thymus, and it is very likely that a general disturbance of mTEC population is involved. The associated disruption of TRA expression, whatever its exact mechanism, is likely to limit the targets of the resulting autoimmunity, but perhaps not the later appearance of additional, less common disease components. Some of the disease manifestations may also reflect the failure of peripheral tolerance, although the significance of peripheral AIRE expression remains poorly understood. Because the relevant tissues cannot be accessed in APECED patients, many of the open questions can be addressed only indirectly. In particular, innovative organ culture methods to analyze the role of AIRE in the human thymus are likely to provide a means to test the proposed pathogenetic pathways.

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The many faces of Aire in central tolerance

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Although the role that Autoimmune Regulator (Aire) plays in the induction of central tolerance is well known, the precise cellular and molecular mechanisms are still unclear and debated. In the prevailing view, Aire serves mainly as a direct inducer of tissue-specific antigens. However, there is a growing amount of evidence suggesting that Aire modulates the differentiation program of medullary thymic epithelial cells, which may directly contribute to the negative selection of self-reactive thymocytes. In addition, Aire has been shown to regulate the expression of many intrathymic chemokines that are required for the proper localization of thymocytes and dendritic cells, and thus are potentially important for direct and indirect self-antigen presentation in the thymic medulla. Further, recent evidence suggests that the induction of certain antigen-specific regulatory T-cells that translocate to tumors and peripheral tissues can be Aire dependent and may contribute to tissue-specific tolerance. This review summarizes the current understanding of the effects of Aire on these alternative mechanisms for the induction of Aire-induced central tolerance.

Keywords: Aire, thymus, Hassall's corpuscle, thymic epithelial cells, central tolerance, chemokines, negative selection, epithelial differentiation

The thymus is the primary lymphoid organ involved in thymocyte development and thus plays a central role in establishing immune tolerance (1). During the course of central tolerance induction, single-positive thymocytes are guided from the thymic cortex to the medulla, tested there for reactivity to self-antigens and, if they are self-reactive, either deleted or directed to become regulatory T-cells (Tregs). Impaired clonal deletion or Treg induction can lead to a breakdown of central tolerance and the development of autoimmune diseases.

AIRE DEFICIENCY RESULTS IN AUTOIMMUNITY

An essential molecule in the induction of central tolerance is Autoimmune Regulator (Aire). The *AIRE* gene was identified by positional cloning of a locus linked to a rare disease, Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) (2, 3). This syndrome usually starts during childhood with chronic mucocutaneous candidiasis, which may correlate with autoantibodies to interleukin (IL)-17 and IL-22 (4). The later stages of this disease are characterized by the presence of autoantibodies to multiple self-antigens and lymphocytic infiltration of various endocrine glands, which finally leads to autoimmune endocrine disorders (5, 6). Although the phenotype of Aire-deficient mice is considerably milder than in APECED and is dependent on the genetic background, it is also characterized by autoantibodies and autoimmune infiltrations, and thus resembles the pathological characteristics of APECED patients (7).

AIRE DEFICIENCY RESULTS IN DEFECTIVE NEGATIVE SELECTION

There is strong experimental evidence that Aire deficiency directly results in the defective negative selection of thymocytes. This evidence comes from transgenic mice in which most of the T-cells express T-cell receptors (TCRs) specific for a certain neo-self-antigen, such as hen egg lysosome (HEL). When this transgenic line is crossed with another transgenic line expressing HEL under the rat insulin promoter [i.e., an RIP-HEL mouse expressing HEL in thymic medullary thymic epithelial cells (mTECs) and in the pancreas], the effectiveness of eliminating autoreactive T-cells by negative selection is strictly dependent on the presence of Aire (8). This role in regulating the thymic clonal deletion of autoreactive thymocytes has also been shown for other neo-self-antigens and for different promoters and clearly indicates a role for Aire in negative selection (9, 10).

AIRE IS PREDOMINANTLY EXPRESSED IN MHC CLASS II-HIGH, CD80-HIGH mTECs

Several studies have expanded our knowledge of Aire and illustrate its key role in central tolerance induction. The majority of Aire signal have been shown to come from mTECs, a very specific set of cells in the thymus (11). mTECs are unique because they can express thousands of tissue-specific self-antigens that are presented to developing thymocytes and are thus associated with negative selection (12). The phenomenon, known as promiscuous gene expression, and the role that Aire plays in this process, have been covered in detail by Ucar et al. in this Research Topic of the Frontiers in Immunology. Within mTECs, Aire expression is specifically located in a subpopulation of cells characterized by the surface expression of MHC class II, and the co-stimulatory molecules CD80 and CD86 (13), indicating that, in addition to antigen cross-presentation by dendritic cells (DCs), Aire+ mTECs also have the potential for direct antigen presentation. Within these MHC class II mTECs, Aire localizes in the nuclei, forming discrete dot-like structures that resemble promyelocytic leukemia (PML) nuclear bodies (11, 14). PML bodies have been associated with several activities, including the modulation of chromatin structure, transcriptional control, DNA repair, and antiviral response (15).

In addition to mTECs, some recent studies have also identified the Aire protein in peripheral lymph nodes both in humans as well as mice (16, 17). It has been shown that the peripheral expression of Aire may contribute to the autoimmune phenotype either via its effects on T-cells as well as directly on B-cells (16, 18). Thus, in addition to its major role in the induction of central tolerance, as is covered in this review, Aire is likely to play a role in peripheral tolerance as well, as has been covered in recent reviews (19).

Thus, in summary, it is well established that (1) the insufficient expression of Aire results in autoimmunity in both humans and mice, (2) Aire is required for negative selection, and (3) the MHC class II-high, CD80-high mTEC population is the primary source of Aire. However, the precise mechanisms that link the expression of Aire in the thymus to the effective induction of tolerance, are still widely debated and are summarized in the remainder of this review (**Figure 1**).

AIRE AND TSA EXPRESSION

The role of Aire as a master regulator of tissue-specific antigens (TSA) was first proposed by Anderson et al. (20) and is by far the best studied and established mechanism behind Aire-induced negative selection. It is clear that Aire controls the expression of many TSAs in mTECs and that Aire-dependent expression of these TSAs leads to the negative selection of self-reactive thymocytes. These aspects of Aire have been covered in depth in recent reviews on Aire (21, 22), and also in a review on promiscuous expression by Ucar et al. in this Research Topic of the Frontiers in Immunology. However, there is also accumulating evidence that the impaired expression of TSAs is not the only mechanism behind Aire-induced central tolerance. For example, Aire-deficient mice develop a Sjögren's syndrome-like autoimmune reaction to α-fodrin, which is a self-antigen not regulated by Aire (23). Similarly, Aire-deficient non-obese diabetic (NOD) mice develop autoimmune pancreatitis to isomerase A2, another Aire-independent self-Ag (24). Therefore, it is clear that Aire has an additional effect on T-cell selection independent of its effect on TSA expression. The precise mechanisms responsible for these additional effects of Aire, however, are still unclear and deserve further studies.

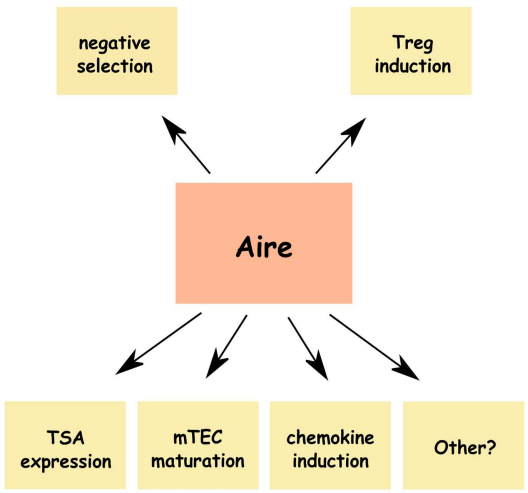

FIGURE 1 | Alternative mechanisms of Aire-induced central tolerance. In addition to the well-described roles in tissue-specific antigen (TSA) expression and induction of negative selection Aire has been shown to affect maturation of medullary thymic epithelial cells (mTECs), expression thymic chemokines, and induction of naturally occurring Tregs, which may all contribute to the induction of central tolerance. Other possible mechanisms, such as induction of mTEC apoptosis or disturbances in thymocyte maturation have also been suggested.

AIRE AND mTEC MATURATION

During maturation, mTECs must pass through several developmental stages that are characterized by the expression of a few key proteins that are related to specific functions at that particular stage of development (**Figure 2**). The classical subpopulations are composed in consecutive order of the (1) MHC class II-low, CD80-low, Aire-mTECs, which are considered to be an immature, highly proliferating population, that is already committed to the mTEC lineage; (2) MHC class II-high, CD80-high, Aire-mTECs, a subpopulation that is already capable of expressing

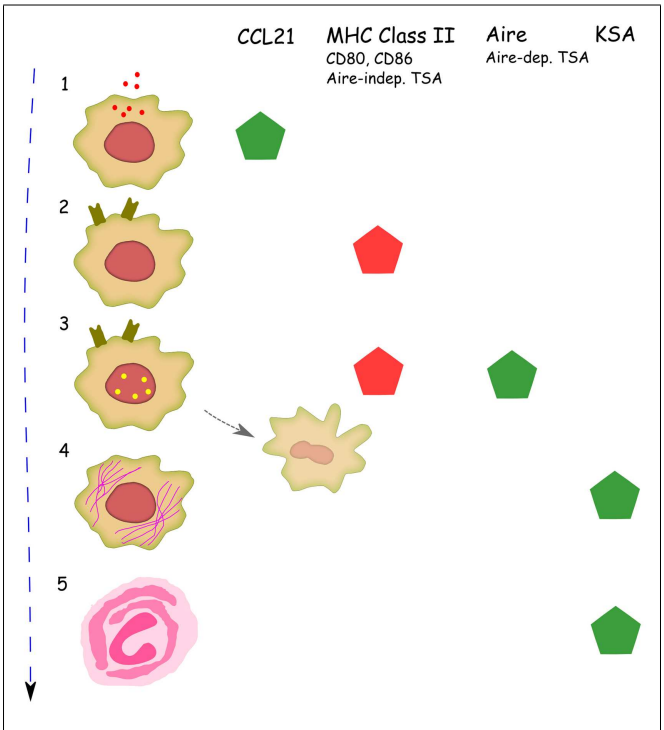

FIGURE 2 | Proposed program of mTEC differentiation and the effect of Aire on mTEC development at different stages of differentiation. Consecutive developmental stages are indicated based on key molecules predominantly expressed at this stage: (1) MHC class II−, CCL21+*; (2) MHC class II+, Aire−; (3) MHC class II+, Aire+; (4) MHC class II-low, Aire−, keratin high; (5) Hassall's corpuscles. Possible pathway from MHC II+, Aire+ cells to apoptosis is shown in pale. The pentagonal signs indicate presence of the specific marker at this stage of differentiation, whereas the color of a sign indicates the change of this marker in the Aire-deficient mice: green corresponds to a decrease and red to an increase in Aire KO mice. TSA stands for tissue-specific antigens and excludes keratinocyte-specific TSAs; KSA stands for keratinocyte-specific antigens. *In parallel to the developmental sequence presented here, the MHC class II−, CCL21+ mTEC stage has been suggested to follow the MHC class II+, Aire+ stage of development (50).

many (Aire-independent) TSAs and directly presenting them to developing thymocytes; and (3) MHC class II-high, CD80-high, Aire+ mTECs. This last cell population consists of matured, post-mitotic mTECs that express a wide variety of TSAs, including the ones under control of Aire (25, 26). Until quite recently, it was widely accepted that these post-mitotic Aire+ mTECs represented the final stage of mTEC maturation. More recent data, however, suggest that the maturation of mTECs may extend beyond the Aire+ stage, and Aire in fact may modulate several different aspects of mTEC differentiation. The first indications that Aire may alter mTEC differentiation came from studies by Gillard et al. and Dooley et al., that characterized the staining patterns of UEA1, Keratin (K)-5/K8/K14, and p63 in Aire-deficient thymi. These authors observed changed proportions of stellate versus globular mTECs and more frequent thymic cysts in Aire-deficient thymi. Although these findings were only apparent after careful analysis, and their functional significance is difficult to evaluate, they clearly suggest that the loss of Aire alters the basic parameters that determine mTEC morphology and maturation stage (27, 28). In addition, a study by Milicevic et al. looked at the ultrastructure of mTECs of Aire-deficient mice using electron-microscopy and found profound changes in all subpopulations of mTECs (29). Rather strikingly, all mTECs in Aire-deficient mice, regardless of their differentiation stage, showed profound ultrastructural changes. These changes were collectively characterized as signs of activation and increased intracellular traffic. Thus, although mTECs classified by either electron-microscopy or functional surface markers are not directly comparable, this ultrastructural study suggested that the effect of Aire on mTEC maturation is not restricted to the final (i.e., Aire+) cells, but rather covers all mTEC subpopulations.

Direct evidence suggesting that mTECs survive and further differentiate after the Aire+ stage originated from recent studies by Yano et al., Nishikawa et al., and Wang et al., all of which used different reporter mice to follow the faith of Aire+ cells (30–32). These studies demonstrated that Aire+ mTECs develop further into MHC class II-low, CD80-low, Aire-mTECs, and thus lose their unique property of direct TSA presentation in parallel with the loss of Aire (31, 32). In addition, the loss of another unique property of Aire+ cells, promiscuous TSA expression, was indicated by the down-regulation of many Aire dependent as well as Aire-independent TSAs in these post-Aire mTECs (32). Instead, post-Aire cells upregulated the expression of keratins and thus came to resemble keratinocytes, at least in terms of the gene-expression pattern (30, 32). Finally, it was shown that the post-Aire cells lose their nuclei and merge to become Hassall's Corpuscles (HC), well known but poorly characterized concentric structures in thymic medullary areas (30, 32). Although the precise function of these structures is unknown, they may have the potential to induce Treg development through expression of TSLP (33). In addition, it has been shown that intrathymic expression of some keratinocyte-specific TSAs, including the expression of the well-known pemphigus-related autoantigens, desmoglein-1 and -3, is clearly restricted to HCs (32, 34–36). Thus, it is plausible that these post-Aire structures contribute to the induction of central tolerance by negative selection of keratinocyte-reactive thymocytes as well as by induction of keratinocyte-specific Tregs.

The lack of Aire seems to block the maturation of mTECs, resulting in the accumulation of MHC class II+, CD80+, truncated Aire+ mTECs, and in a severe reduction in the expression of many keratins and the numbers of HCs (28, 31, 32) (**Figure 2**). The reduction in the expression of keratinocyte-specific proteins in Aire-deficient thymi involves also desmoglein-3 and results in the altered selection of desmoglein-3-specific T-cells and defective tolerance against desmoglein-3 (37). Thus, it has been formally demonstrated that through its effect on mTEC differentiation, Aire can promote tolerance at least against this specific keratinocyte-related TSA. Whether, and to what extent, the broad effect of Aire on gene expression is regulated through mTEC differentiation rather than direct transcriptional activity, remains to be determined in future studies.

AIRE AND CHEMOKINE EXPRESSION

The negative selection of developing thymocytes is also dependent on coordinated migration through distinct thymic niches, that provides timely interactions with mTECs and DCs (38). Cell–cell contact with TSA-expressing mTECs requires that the positively selected thymocytes migrate from the thymic cortex to the medulla, whereas indirect TSA presentation is likely to be dependent on the physical proximity of thymocytes, mTECs and DCs (39) (**Figure 3**). The ligands of two chemokine receptors, CCR7 and CCR4, have been previously associated with thymocyte migration to the site of negative selection. Both receptors are predominantly expressed on double positive (DP) and single-positive (SP) CD4-thymocytes (40–42), while the ligands for CCR7 and CCR4 are produced predominantly in the thymic

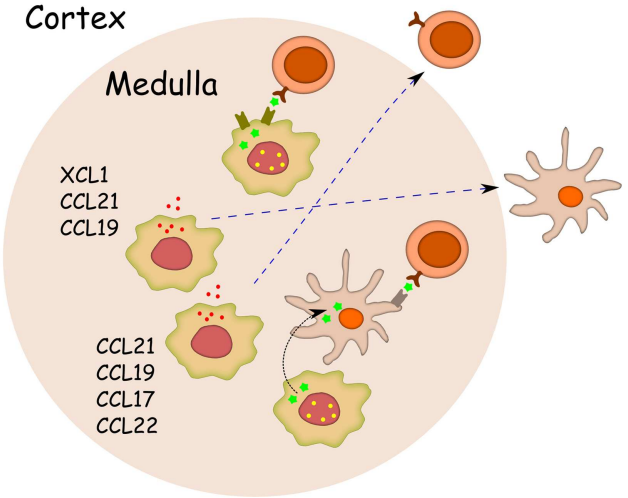

FIGURE 3 | Aire regulates chemokines required for proper localization of thymocytes and dendritic cells. For effective direct antigen presentation (shown as green pentagons) from mTEC to thymocyte, the positively selected single-positive thymocytes need to migrate from cortex to medulla. For effective indirect antigen presentation both, the positively selected thymocytes and the DCs, need to migrate to medulla. Only the key chemokines (shown as red particles) known to mediate the migration of single-positive thymocytes or DCs, and also known to be regulated by Aire (shown as yellow dots) are listed.

medulla (40, 41, 43–45). The importance of CCR7 ligands in thymocyte development has been further highlighted in the CCR7-deficient mouse, in which the impaired intrathymic migration of thymocytes resulted in a delay of mature T-cell emigration and a leakage of premature T-cells (45). Significantly, a lack of CCR7 caused defective induction of central tolerance and the presence of multiple autoantibodies and autoimmune infiltrations in many peripheral organs, resulting in a phenotype almost identical to that of Aire-deficient mice (46, 47). In turn, the importance of Aire in the regulation of thymic chemokine expression has been reported in many studies. First, microarray analysis of sorted mTECs has demonstrated the down-regulation of several chemokines in the Aire-deficient mouse (10). Second, the effect of Aire on CCR4 and CCR7 ligand expression has been further validated with both the up- and down-regulation of Aire (48, 49). Third, the delay of thymocyte emigration (in a manner similar to that in CCR7-deficient mice) is also present in the Aire-deficient mice (48). Notably, the highest expression of CCR7 ligands, although Aire dependent, occurs in the MHC class II-low mTECs, i.e., in a population not expressing Aire (48, 50). Further, during mouse ontogeny, CCL21+ (i.e., CCR7 ligand-producing) cells appear after the appearance of Aire+ cells (50). It has therefore been proposed that MHC class II-, CD80−, CCL21+ mTECs represent the post-Aire population (50). However, we have measured the levels of multiple chemokines directly in the post-Aire population in the Aire-reporter mouse, and found a clear down-regulation of all measured chemokines in these post-Aire cells (32). Therefore, although the precise developmental sequence is still clearly under debate, we feel that the MHC class II-, CD80− population prior to the induction of Aire is a more likely source of CCR7 ligands (**Figure 2**). It remains unclear, however, how the expression of Aire in one cell can influence another mTECs in a less mature stage. In addition to possible direct paracrine signaling from mTEC to mTEC, another possibility involves cross-talk between mTECs and thymocytes, which do not receive all appropriate signals from the mTECs under Aire-deficient situation, resulting in improper signaling from thymocytes back to immature mTECs. This effect of Aire on mTEC maturation and differentiation before and after Aire expression is in agreement with the ultrastructural changes observed during all stages of mTEC development that have been discussed above (29).

In addition to its effect on thymocyte migration, Aire expression in mTECs has been shown to regulate the chemokines responsible for DC migration. Although not characterized in the thymus, peripheral DCs bear the CCR7 receptor, which is required for their proper localization within lymph nodes (51). In addition, it has been shown by Lei et al. that thymic DCs express the receptor for the chemokine XCL1, which is specifically expressed by mTECs in an Aire-dependent fashion, and is required for the proper localization of DCs to the cortico-medullary junction (49). Thus, there is evidence that Aire-dependent chemokines are required for thymocyte and DC migration to the location where antigen (cross)-presentation and negative selection are likely to occur (**Figure 3**). In fact, altered cross-presentation in *Aire* KO mice has previously been demonstrated (52), although the roles of specific chemokines in this process are still unclear.

AIRE AND Treg INDUCTION

There is an increasing amount of direct evidence that, in addition to their proposed role in negative selection, mTECs contribute to central tolerance by inducing naturally occurring Tregs (53, 54). Accordingly, since the characterization of an autoimmune phenotype in Aire-deficient mice, a significant number of studies have focused on the potential role of Aire in thymic Treg induction. However, there was no major change in either Treg numbers or function in transgenic models (RIP-HEL, RIP-mOVA), in which the negative selection of neo-self-antigen-specific thymocytes was clearly Aire dependent (8–10). Likewise, the lack of Aire had no major effect on TCR usage by Foxp3+ Tregs, in a study where the effect of Aire on Treg TCR repertoire was assessed in a mouse model with restricted TCR repertoire (55). Thus, initial studies that looked at the total numbers and/or function of Tregs, indicated that defects in central tolerance in Aire-deficient mice are not Treg related. Nevertheless, a recent study showed that Aire expression is required for the intrathymic production of tumor-specific Tregs in a mouse model of oncogene-driven prostate cancer (56), and clearly demonstrated a role for Aire in induction of a specific population of naturally occurring Tregs. Thus, this study demonstrates directly that the key role of Aire in central tolerance is not limited to its effect on negative selection but also includes its effect on thymic Treg induction. Future studies will determine, whether this intriguing effect of Aire extends to a significant pool of TSA-specific Treg induction and, if so, what mechanisms cause this effect.

OTHER PLAUSIBLE MECHANISMS BEHIND AIRE-INDUCED CENTRAL TOLERANCE

There are also a number of studies indicating that in addition to the mechanisms covered above, Aire may influence other basic mechanisms, directly in mTECs or indirectly in other thymic cells which, at least hypothetically, may contribute to defects in negative selection.

Thus, Aire may play a role in the induction of apoptosis, as the over-expression of Aire results in the induction of apoptosis in several *in vitro* cell-lines (25, 57, 58). Based on these data, it has been proposed that the absence of this effect is responsible for the increased numbers of MHC class II+ mTECs observed in Aire-deficient mice and that Aire-induced apoptosis may facilitate the cross-presentation of TSAs expressed by these apoptotic cells to the thymic DCs (25, 28). As the apoptotic processes in the thymus are very dynamic and thus difficult to monitor, this attractive hypothesis has not yet been validated *in vivo*. It is, however, completely plausible that, in addition to the maturation of post-Aire mTECs and HCs, a subpopulation of Aire+ mTECs are directly guided to undergo apoptosis and are then quickly removed by resident macrophages (**Figure 2**).

In addition, a report by Li et al. shows that the development of SP CD4-thymocytes is blocked at the transition from SP3 to SP4 in Aire-deficient mice (59). Although this may be due to imperfect cross-talk between defectively matured Aire-deficient mTECs and developing thymocytes, or to insufficient cell–cell contact as a result of reduced chemokine expression, neither of these possibilities has been formally tested. Additionally, the functional consequences of this phenomenon remain unknown.

In summary, although the effects of Aire on central tolerance are well established, the cellular and molecular mechanisms are still unclear. Along with the better-understood effects on TSA expression, Aire can also alter the differentiation program of mTECs, regulate the expression of thymic chemokines, contribute to specific Treg induction, and induce mTEC apoptosis. It remains to be determined, however, what extent these alternative mechanisms contribute to the autoimmune phenotype observed in Aire-deficient mice and APECED patients.

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APECED: a paradigm of complex interactions between genetic background and susceptibility factors

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Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autosomal recessive disease, caused by mutations of a single gene named Autoimmune regulator gene (AIRE) which results in a failure of T-cell tolerance. Central tolerance takes place within the thymus and represents the mechanism by which potentially auto-reactive T-cells are eliminated through the negative selection process. The expression of tissue-specific antigens (TSAs) by medullary thymic epithelial cells (mTECs) in the thymus is a key process in the central tolerance and is driven by the protein encoded by AIRE gene, the transcription factor autoimmune regulator (AIRE). A failure in this process caused by AIRE mutations is thought to be responsible of the systemic autoimmune reactions of APECED. APECED is characterized by several autoimmune endocrine and non-endocrine manifestations and the phenotype is often complex. Although APECED is the paradigm of a monogenic autoimmune disorder, it is characterized by a wide variability of the clinical expression even between siblings with the same genotype, thus implying that additional mechanisms, other than the failure of Aire function, are involved in the pathogenesis of the disease. Unraveling open issues of the molecular basis of APECED, will help improve diagnosis, management, and therapeutical strategies of this complex disease.

Keywords: autoimmune polyglandular syndrome type 1, APECED, autoimmune regulator gene, phenotypic variability, tolerance

INTRODUCTION

Autoimmune Polyglandular Syndrome Type 1 (APS-1), also called Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a rare autosomal recessive disease caused by mutations of the autoimmune regulator gene (AIRE). Immunologically, APECED is characterized by destruction of the target organs by a cellular- and/or antibody-mediated attack (1). In the past decade, much interest has been focused on the pathogenesis of this syndrome. Indeed, APECED represents a paradigm of genetically determined systemic autoimmunity. However, the great variability that characterizes APECED, irrespectively of the AIRE genotype, implies that several factors are involved in the disease phenotypic expression.

In this review, we will focus on the complex pathogenesis of APECED and on the potential interfering factors involved in the clinical expression of the disease.

THE BASIS OF THE IMMUNOLOGICAL TOLERANCE

Tolerance represents a state of immunologic non-responsiveness in the presence of a particular antigen. In this context, T-cell tolerance is crucial for the creation of a proper T-cell repertoire, able to respond to a huge number of foreign antigens, but preventing autoimmune reactions. Imposition and regulation of self-tolerance within the T-cell repertoire is exerted at two levels: (1) central tolerance (development and selection of T-cells in the thymus) and (2) peripheral tolerance (deletion, anergy of mature T-cells in lymphoid and non-lymphoid organs) (2).

T-cell central tolerance, established within the thymus, mostly relies on two main mechanisms: negative selection, also referred to as clonal deletion of maturing thymocytes and positive selection of maturing T-cells able to bind to a surface major histocompatibility complex (MHC) molecule with mild threshold of reactivity (**Figure 1**). The thymus provides the necessary environment for thymopoiesis and establishment and maintenance of self-tolerance (3–5). Thymus contains thymic epithelial cells (TECs) that form a complex three-dimensional network organized in cortical and medullary compartments (6). On entering the thymus, immature thymocytes promote the differentiation of precursor thymic epithelial cells (pTECs) into cortical TECs (cTECs) and medullary TECs (mTECs), playing an important role in the formation of the thymic microenvironment (7–9). During postnatal life, hematopoietic progenitors enter the thymus from the bloodstream (10) and cells committed to the T lineage undergo division, mostly within the double-negative (DN) stage of the T-cell development. The first checkpoint is the rearrangement of T-cell receptor (TCR) β and α locus. Expression of αβ TCR heterodimers on the cell surface allows DN thymocytes to progress to the double-positive (DP) CD4+CD8+ stage. At DP stage, the TCR affinity for self-peptide-MHC on mTECs within the thymus determines thymocyte's fate. mTECs express a wide array of tissue-specific antigens (TSAs) in the context of MHC class II molecules; these TSAs include self-proteins derived from different organs in the body. DP thymocytes expressing TCRs that do not bind self-peptide-MHC complexes are programed to undergo "death by

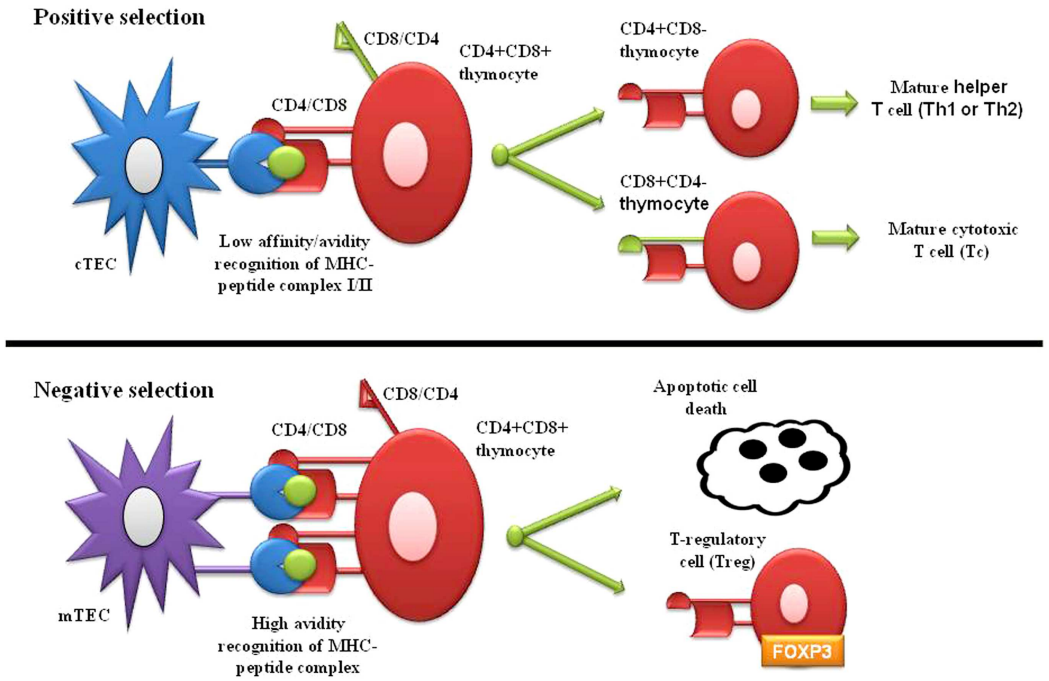

FIGURE 1 | Positive and negative selection of immature thymocytes within thymus.

neglect" or apoptosis. Only about 5% of DP has a low affinity for self-peptide-MHC complexes and differentiate to CD4+CD8− or CD4−CD8+ single positive (SP) lineage (positive selection) (11–13). DP thymocytes with high-affinity TCR for MHC complexes represent a potential reservoir of auto-reactive lymphocytes and "clonal deletion" (negative selection) is the main mechanism in the thymus to preserve self-tolerance (14, 15). Compelling evidence indicates that an altered promiscuous thymic expression of TSAs leads to autoimmunity. In the autoimmune attack, T helper cells (Th) escaped to self-tolerance, produce pro-inflammatory cytokines able to begin inflammation and activate auto-reactive B-cells, resulting in autoantibodies production, which lead to tissue inflammation and damage (1). Some of the thymocytes that recognize self-peptide-MHC complexes with high-affinity express Foxp3 and through "clonal diversion" mature as regulatory T-cells (Tregs), which are able to suppress auto-reactive T-cells in the periphery (16–18). The central tolerance is not able alone to completely remove mature T-cells with self-antigens specificity, therefore additional mechanisms in the periphery are also needed to maintain immunological tolerance.

The peripheral tolerance recognizes as possible mechanisms the induction of functional anergy, deletion of auto-reactive clones, and the suppressive action of T-regulatory cells (Tregs). Anergy is a state of long-term hyporesponsiveness with inactivation of self-reactive T-cells in the presence of a TCR signal but in the absence of a second costimulatory signal, necessary to T-cell activation. Deletion of self-reactive lymphocytes is achieved in both the thymus and the periphery by apoptosis through interaction of Fas/FasL. The function of Tregs (Foxp3-expressing CD4 T-cells) is to suppress immune responses through numerous mechanisms including the production of anti-inflammatory cytokines, direct cell–cell contact, and by modulating the activation state and function of antigen-presenting cell (APC) (19). An additional mechanism involved in controlling reactivity to self in the periphery is NK cell activity.

AIRE AND THE MAINTENANCE OF IMMUNOLOGICAL TOLERANCE

Autoimmune regulator gene encodes for a transcription factor (Aire) involved in the maintenance of tolerance. In humans, the AIRE gene maps to chromosome 21q22.3 (20, 21). It consists of 14 exons spanning 11.9 kb of genomic DNA (22) and encodes a 545 amino acid protein with a molecular weight of 58 kDa that works as a "non-classical" transcriptional factor in immune-related organs. The highest level of AIRE expression has been detected within the thymus (23) in mTECs, followed by thymic dendritic cells (DCs). In addition to the thymus, low level of Aire seems to be expressed in secondary lymphoid organs, such as lymph nodes, fetal liver, and spleen (24, 25). The Aire protein, mostly localized in the cell nucleus, is composed by specific domains including the amino-terminal HSR domain, the nuclear localization signal (NLS), the Sp100, AIRE1, nucP41/75, DEAF 1 (SAND) domain, two plant homeodomain (PHD) type zinc fingers, and four LXXLL motifs (26) (**Figure 2**). The HSR region has been shown to be responsible for the dimerization of the polypeptides belonging to the Sp100 protein family (27). The SAND domain is important for AIRE transactivation capacity and subcellular localization. The PHD zinc fingers are often found in proteins involved in the regulation of transcription (28). The LXXLL motifs are found on coactivators nuclear receptors and proline-rich regions (PRR) and are also

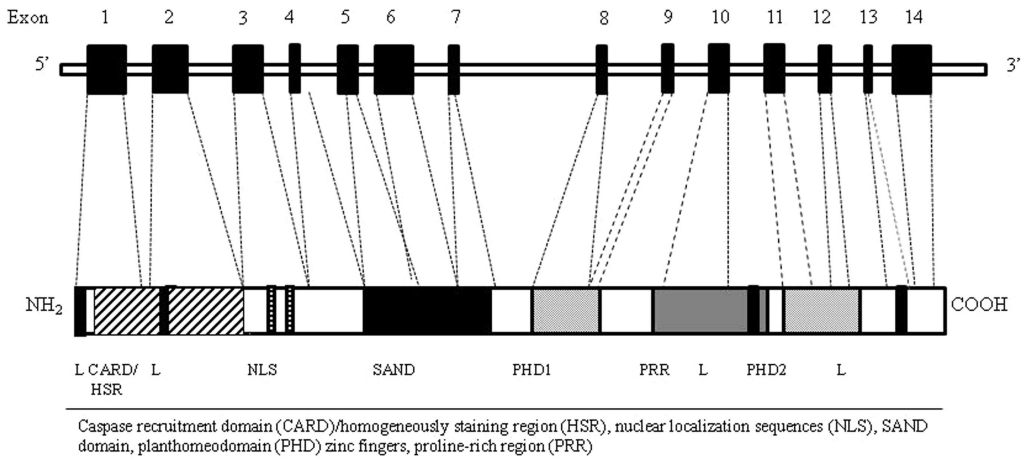

FIGURE 2 | AIRE gene (top) and corresponding protein (bottom) with functional domains. The Aire protein is composed by nuclear localization sequences (NLS), two planthomeodomain (PHD) zinc fingers, a caspase recruitment domain (CARD)/homogeneously staining region (HSR), a SAND domain, and a proline-rich region (PRR).

associated to transcription regulation (29). Although the precise molecular mechanism is still unclear, Aire seems to regulate the transcription process acting as a coactivator in a large transcriptional complex (30), and interacting with a large set of partners, divided into four main classes based on their function: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing factors (31). The first protein reported to bind to AIRE was CREB-binding protein (CBP) (32). Its interaction with AIRE may lead to promotion of gene transcription through histone acetylation and the recruitment of chromatin-transcription factors (33). Other AIRE partners have been identified, such as DNA protein kinase (DNA-PK), SP-RING domain protein inhibitor of activated STAT1 (PIAS1), positive transcription elongation factor b (P-TEFb) (34–36). Moreover, it has been proposed a possible epigenetic control of the AIRE target genes since AIRE's PHD1 finger domain appears to be able to bind histone three molecules with unmethylated lysine at position 4, generally associated with repressed genes (37). Overall, it is possible that Aire mediates the expression of TSA in mTECs through its co-transcriptional partners (38). The intriguing question is how the AIRE gene alone can influence the transcription of such a large number of TSA genes. Indeed, two models have been suggested to explain the action of Aire: transcription model and maturation model. In the transcription model, TSAs are considered to be the direct target genes of Aire's transcriptional activity and the lack of Aire protein within the cell would result in the defective TSA gene expression, while the maturation program of mTECs would be in principle unaffected. The maturation model suggests that Aire may affect the thymic microenvironment more globally than through simple control of TSA expression levels. Consequently, in keeping with the latest model the regulation of TSA gene expression might not be the major defect of Aire-deficient mTECs responsible for impaired negative selection (39).

Although the exact role of AIRE in controlling T-cell tolerance is still largely unclear, several mechanisms have been suggested. Functional alterations of AIRE may affect processing and/or presentation of self-antigens within the mTECs (40). The process of thymocyte maturation (41), the attraction of mature thymocytes to their final location for a proper negative selection (40, 42), the control of cross-presentation through alteration of the relationship between APCs and mTECs (43) may also represent potential mechanisms by which AIRE alterations may lead to functional abnormalities of the central tolerance. The alteration in the balance between negative selection and regulatory T-cell production (44) may also be implicated in the pathogenesis. In addition, Aire may also play a role in the proper differentiation of the thymic medullary epithelium, in the induction of apoptosis in end-stage terminally differentiated mTECs (39) as well as in mTECs' differentiation program. In particular, evidence suggests that lack of Aire in mTECs results in an arrest of the differentiation program, with the cells remaining at the premature stage just before terminal differentiation (45, 46).

THE CLINICAL COUNTERPART OF AIRE MUTATION: APECED

GENETIC BACKGROUND

Mutations in AIRE gene result in development of APECED, which represents the paradigm of a genetically determined failure of central tolerance leading to autoimmunity (46). APECED is a rare autoimmune syndrome, but it has been reported worldwide showing a relatively higher prevalence in genetically isolated populations such as Iranian Jews (1:9,000) (47), Finns (1:25,000) (48, 49), and Sardinians (1:14,400) (50). It is also quite frequent in Norway (1:90,000) (51) and in some regions of Italy (52–55). The most frequent model of inheritance is autosomal recessive, even though a dominant pattern has also been sporadically reported (56). So far, over 70 different mutations of AIRE have been documented (2). Due to the molecular organization and the complexity of intermolecular connection of Aire, it would be expected that different mutation in the molecule might imply different functional abnormalities, thus being associated with a variable phenotypic expression. Single nucleotide substitutions, small insertions, deletions, and mutations affecting splice

consensus sequences have been identified along the entire coding region, and include either nonsense or frameshift mutations that result in truncated polypeptides, or missense mutations that result in single amino acid-changing (27). Most of these AIRE mutations lead to a change in its subcellular location altering the distribution of the protein between the nucleus and cytoplasm (27). Mutations of the predicted surface area of the HSR domain cause the protein accumulation in the nucleus blocking its cytoplasmic localization probably enhancing nuclear import or inhibiting nuclear export (57). Mutations of the SAND domain disturb the distribution of Aire between the nucleus and cytoplasm suggesting a role for the SAND domain in nuclear transport mechanisms (57). Moreover, since the six helix CARD domain is involved in homodimerization, missense mutations in this region often affect Aire multimerization or localization to nuclear bodies (58) while most of the missense mutations in PHD domains alter the zinc-finger fold and decrease Aire's transcriptional activation capacity (38).

Some different mutations have been found to be peculiar to certain populations. R257X is the most common mutation among Finnish and other European patients (59–61). R257X is a nonsense mutation, which most probably results in a carboxy-terminally truncated, non-functional Aire protein leading to alterated subcellular localization and inhibition of the transactivation function and complex formation of Aire (27). The 1094–1106 del113 (or 967–979 del-13 bp) is the most common mutation in British (62), Irish (63), North American (64, 65), and Norwegian patients (51) leading to the truncation and loss of function of Aire. Y85C is the only missense mutation found among Iranian Jews (57). In Italy, typical mutations of AIRE have been detected in Sardinia (R139X on exon 3) (50, 54), where this nonsense mutation leads to a total absence of Aire and seems to be associated with a more severe phenotype. In Apulia, the missense mutation W78R on exon 2, and the nonsense mutation Q358X on exon 9 have been found. The mutation Q358X lies in the PRR resulting in a truncated protein which lacks the second PHD finger and thereby is most likely non-functional protein (53). In Sicily, the most frequent mutation is R203X on exon 5, and two novel mutations, S107C and Q108fs on exon 3, have been detected. The mutation S107C is a missense mutation, whilst Q108fs is a small deletion, both affecting the HSR domain of Aire protein and it is likely that the Aire protein loses its homodimerization properties (66, 67). In Venetian patients, the most frequent mutations are R257X on exon 6 and 979 del-13 bp on exon 8, that are analogous to those detected in Finnish and Anglo-saxon patients but different from Italian ones (55). No typical mutations have been identified neither in Calabria nor in Campania (52, 68) even though patients from Campania show a high frequency of mutations in the exon/intron 1 junction. Compared to other mutations, the R257X results in a total loss of function, whereas the less dramatic truncations of the AIRE protein and many missense mutations, especially the predicted surface mutations of the HSR domain and the mutations in the leucine zipper domain, seem to exert less severe effects on the function of the Aire protein (27). Therefore, despite considerable variations in the APECED genotype, correlations with specific phenotypic features are far from being well elucidated. Only in patients affected with Candida infection, a correlation has been proved. In fact, candidiasis was significantly less prevalent in patients homozygous for 967–979del-13bp than in patients carrying the R257X or R139X, suggesting that AIRE truncation upstream the SAND domain promotes the susceptibility to this infection (69).

DIAGNOSIS OF APECED

The onset of APECED usually occurs during childhood. The clinical diagnosis is based on the presence of two of the three classical components: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison's disease (AD). The presence of only one of these features is sufficient for the diagnosis, when a sibling is affected. Molecular analysis of AIRE may help to confirm the clinical diagnosis, in particular in those cases with an atypical presentation (70, 71). Neutralizing autoantibodies against IFN-ω and IFN-α may represent a precocious biomarker detectable in the majority of patients and, thus they have been recently included in the diagnostic criteria of APECED (72).

CLINICAL EXPRESSION, AUTOANTIBODIES PROFILE, AND SUSCEPTIBILITY FACTORS

APECED is characterized by a highly variable pattern of destructive autoimmune reaction, mainly mediated by specific autoantibodies toward different endocrine and non-endocrine organs. Virtually, all tissues and organs may represent the target of the autoimmune attacks, thus leading to a wide spectrum of clinical features. As already mentioned, the three main components of APECED are CMC, CH, and AD. CMC is, generally, the first component to develop, often followed by CH, before the age of 10 years and later by adrenal insufficiency (73, 74). In addition to the main components, the spectrum of minor manifestations may include ectodermal dystrophy, other endocrinopathies, such as hypergonadotripic hypogonadism, insulin-dependent diabetes, autoimmune thyroiditis, and pituitary dysfunction. Moreover, gastrointestinal disorders (chronic atrophic gastritis, pernicious anemia, malabsorption, autoimmune hepatitis and cholelithiasis), skin diseases (vitiligo and alopecia), keratoconjunctivitis, immunological defects, asplenia may be present (70). More rare manifestations of the disease include immune-mediated central and peripheral neurological manifestations, such as chronic inflammatory demyelinating polyneuropathy (54) and posterior reversible encephalopathy syndrome (PRES) (75), tubulointerstitial nephritis, autoimmune bronchiolitis, reversible metaphyseal dysplasia, hypokalemia, and hypertension (72).

The majority of APECED components have been correlated with specific autoantibodies that may represent an useful tool for the diagnosis and the follow-up of patients (**Table 1**). Autoantibodies' profile may parallel clinical expression even though a strong correlation with the phenotype and the severity of the disease is not always present. Indeed, only some autoantibodies are highly predictive of specific organ's failure, being detectable years before the onset of the overt clinical manifestations.

APCED-related CMC has been associated with the presence of specific autoantibodies against the Th17-related cytokines interleukin- (IL-) 22 and IL-17F (76, 77). A parathyroid-specific autoantigen called NACHT leucine-rich-repeat protein 5 (NALP5), which is expressed in the cytoplasm of the main cell type in the parathyroid glands (78), has been recently proposed as the immunological hallmark of APECED-related CH.

Table 1 | Clinical counter part of autoantibodies profile in APECED [modified by Capalbo et al. (73)].

Clinical features	Autoantibodies
CMC	Abs against IL-22, IL-17F, and myosin-9
ENDOCRINE MANIFESTATIONS	
HP	Abs against NALP5
AD	Abs against CYP21, CYP11A1, CYP17
Ovarian failure	Ab against CYP11A1, CYP17, and NALP5
Type 1 diabetes	Ab against IA-2 and insulin
Autoimmune thyroiditis	Ab against TPO and Tg
NON-ENDOCRINE MANIFESTATIONS	
Ectodermal manifestations	
Vitiligo	Abs against Melanocytes, SOX-9, SOX-10, and AADC
Alopecia	Abs against TH
Gastrointestinal manifestations	
Autoimmune gastritis/ pernicious anemia	Abs against parietal cells and IF
Autoimmune hepatitis	Abs against CYP-1A2, CYP-2A6, AADC, and TPH
Autoimmune enteropathy	Abs against TPH, HD, and GAD
Rare manifestations	
Pulmonary disease	Abs against KCNRG
Demyelinating polyneuropathy	Abs against myelin protein zero
Tubular interstitial nephritis	Abs against proximal tubule
Non-organ specific Abs	Abs against IFN-α and IFN-ω

Abs, autoantibodies; IL-17F, interleukin 17F; IL-22, interleukin 22; NALP5, NACHT leucine-rich-repeat protein 5; CYP21, 21-hydroxylase; CYP11A1, cholesterol side-chain cleavage enzyme; CYP17, 17-α-hydroxylase; IA-2, tyrosine phosphatase-like protein; TPO, thyroid peroxidase; Tg, thyroglobulin; AADC, aromatic L*-amino acid decarboxylase; TH, tyrosine hydroxylase; IF, intrinsic factor; TPH, tryptophan hydroxylase; HD, histidine decarboxylase; GAD, glutamic acid decarboxylase; CYP-1A2, cytochrome P450 1A2; CYP-2A6, cytochrome P450 2A6; KCNRG, potassium channel-regulating protein; IFN-α, interferon α; IFN-ω, interferon ω.*

Antibodies against the enzyme 21-hydroxylase (CYP21) are strongly associated and highly predictive for the development of AD in patients with CH and/or CMC (79, 80). Steroidogenic enzymes such as Cholesterol side-chain cleavage enzyme (CYP11A1) and 17α-hydroxylase/17,20-lyase (CYP17) represent a further targets of autoimmune reaction against adrenal cortex, moreover they are highly correlated with ovarian insufficiency due to lymphocytic oophoritis and can precede the clinical onset of the component (81, 82). Autoimmune gastritis is associated with the presence of autoantibodies against parietal cells and intrinsic factor (IF), the latter being involved in the development of pernicious anemia (83). The presence of autoantibodies against tryptophan hydroxylase (TPH), an enzyme involved in the synthesis of neurotransmitters in the nervous system and in the gastrointestinal endocrine cells correlates with Autoimmune enteropathy (84–87). Moreover, autoantibodies against both histidine decarboxylase (HD), an enzyme expressed in entero-chromaffin-like cells, and GAD (88, 89) have been associated with an autoimmune intestinal involvement. AH is mainly associated with the presence of autoantibodies against cytochrome P4501A2 (CYP-1A2), CYP-2A6, and aromatic L-amino acid decarboxylase (AADC), even though other types of autoantibodies, such as those directed against TPH, have been correlated with the AH component of the APECED phenotype (54, 90–92). Complement-fixing melanocyte autoantibodies and antibodies against transcription factors SOX-9, SOX-10, and AADC (83, 89) and tyrosine hydroxylase (TH) strongly correlate with the presence of vitiligo and alopecia (72, 83). Recently, several reports have confirmed an important role of autoantibodies against IFN-α and IFN-ω, which, although not tissue-specific, have been detected in the serum of almost all APECED patients (93, 94). Furthermore, they appear at a very early stage, often before the onset of any clinical manifestation. With this regard, their presence may be considered as an additional diagnostic marker of the disease, especially in those cases with an atypical presentation (94, 95). Although autoantibodies' production seems to be a key-event in the development of the clinical disease, their role in the pathogenesis of APECED still remains to be defined.

APECED is a paradigmatic example of an autoimmune monogenic disease, however, the phenotypic presentation can widely vary from one patient to another (67, 70, 96, 97). Indeed, there are observations documenting a genotype-phenotype correlation only for specific traits (98, 99), but a clear genotype-phenotype correlation is lacking. We have, recently, reported on a family with an extremely wide intra-familial clinical variability despite the same mutation of AIRE (100). These observations suggest that genetic background is not able to explain alone the variability of the clinical expression and the severity of APECED and that, as for other monogenic diseases, the phenotypic variability of the syndrome may result from the complex interaction between several genetic, epigenetic, immunological, and/or environmental factors. The HLA class I and class II alleles have been reported to confer susceptibility to develop autoimmune diseases, such as Type 1 diabetes and autoimmune thyroid diseases (101). Only few studies investigated the association between the APECED phenotype and HLA genotypes, reporting conflicting results. In fact, although some studies did not find any significant association between HLA antigens class I or II and autoantibodies' production or clinical expression of the disease (74, 102–104), other showed an increased frequency of specific HLA genotypes in APECED patients (105). However, in a more recent study on 18 Sardinian patients (54) autoimmune hepatitis, as well as LKM autoantibodies, have been found to be strongly associated with HLA-DRB1*0301/DQB1*0201. However, there is no evidence indicating that the HLA haplotype might be associated to a particular severity of the disease. Infectious agents are potent stimuli for the immune system, and thus both viruses and bacteria can be considered as trigger of an autoreaction via different mechanisms, such as molecular mimicry, bystander activation, and epitope spreading (106–111). Moreover, evidence suggests that a genetically determined susceptibility may favor the development of an autoimmune disorder after an infection. Many viruses have also been proposed as factors exacerbating several autoimmune processes (112). However, the role of the infectious

triggers has not been sufficiently investigated in patients with APECED, and preliminary results did not show any significant effect of different infections on the phenotypic expression of the syndrome (100). As already mentioned, along with the central tolerance network, which is primarily involved in the pathogenesis of APECED, several peripheral mechanisms are capable of contributing to the control and regulation of the immune system. These factors are involved in maintenance of the homeostasis by controlling residual auto-reactive clones, which escape negative selection within the thymus and play a significant role in preventing or minimizing reactivity to self-antigens. The peripheral tolerance recognizes as possible mechanisms the induction of functional anergy with inactivation of self-reactive T-cells, deletion of auto-reactive clones by apoptosis, through Fas/FasL interaction, and the suppressive action of Tregs. An additional mechanism involved in controlling reactivity to self engages in the periphery is represented by NK cell activity. A possible role of altered peripheral tolerance in the pathogenesis and clinical expression of APECED might be hypothesized also considering that recent evidence suggesting that Aire may also be implicated in the control of peripheral mechanisms dedicated to the peripheral maintenance of self-tolerance. In the periphery, Aire is expressed in DCs and a specific population of extrathymic Aire-expressing cells (113, 114). As in the thymus, also in secondary lymphoid organs Aire is required for the expression of many TSAs. However, only few studies investigated the functionality of peripheral tolerance mechanisms in patients with APECED and the role of a failure in the peripheral mechanisms of Aire's function is still poorly defined. Studies on animal models of APECED suggest that Aire does not influence *per se* Tregs as in Aire-KO mice the number of CD4+CD25+ cells are normal, and the functionality in *in vitro* suppression assays is normal as well (115, 116). However, the link between Aire and Treg cells is still not fully understood. Some recent studies suggest that Aire-expressing mTECs are involved in the generation of TSA-specific Foxp3+ Treg cells. A recent study supports this concept by showing that Aire-expressing mTECs, in addition to providing an antigen reservoir, also serve as APCs, thus enhancing the selection of Treg cells. The commitment of Tregs was shown to occur independently of Foxp3, and interaction of developing thymocytes with thymic stromal cells may drive the differentiation of a thymocyte subpopulation into the Treg cell lineage and, subsequently, trigger the expression of Foxp3 (117). Some adult APECED patients have lower proportion of Tregs (118), this finding being probably related to chronic infections, to the extent of autoimmune inflammation or therapy. Unfortunately, Tregs have been evaluated in only two children with APECED. Although in these children the number of Tregs was reduced in comparison to healthy controls, confirming the results obtained in adult patients, this reduction was not related to the severity of the disease, thus ruling out a potential role in modulating the clinical expression of the syndrome (100).

CLOSING REMARKS

Although APECED is a monogenic autoimmune disease, the great variability of the clinical expression and the absence of a clear genotype-phenotype correlation implies that, beyond AIRE mutations, other susceptibility factors such as immunological and environmental factors may be involved in the pathogenesis of the disease. The evidence of a role of an impairment of central and peripheral tolerance and of other susceptibility factors in the phenotypic variability of APECED is limited and needs to be further investigated. So far, the reason of such variability still remains obscure. Unraveling the open issues of the molecular basis of APECED, will be extremely useful in improving the diagnosis, management, and therapeutical strategies of this complex disease. As for other Mendelian diseases, total exome sequencing could be a good perspective to analyze other genetic variations and to identify potential disease-modifying genes involved in the clinical expressivity of organ-specific autoimmunity.

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Immunodeficiency in DiGeorge syndrome and options for treating cases with complete athymia

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The commonest association of thymic stromal deficiency resulting in T-cell immunodeficiency is the DiGeorge syndrome (DGS). This results from abnormal development of the third and fourth pharyngeal arches and is most commonly associated with a microdeletion at chromosome 22q11 though other genetic and non-genetic causes have been described. The immunological competence of affected individuals is highly variable, ranging from normal to a severe combined immunodeficiency when there is complete athymia. In the most severe group, correction of the immunodeficiency can be achieved using thymus allografts which can support thymopoiesis even in the absence of donor-recipient matching at the major histocompatibility loci. This review focuses on the causes of DGS, the immunological features of the disorder, and the approaches to correction of the immunodeficiency including the use of thymus transplantation.

Keywords: DiGeorge syndrome, immunodeficiency, thymus transplantation, 22q11 deletion, T-cell development

INTRODUCTION

DiGeorge syndrome (DGS) was first described in the 1960's and classically comprises T-cell deficiency (due to thymic hypoplasia), hypoparathyroidism, cardiac malformations, and facial abnormalities. Subsequently, it was recognized that deletions of the long arm of chromosome 22 at position q.11 were most commonly associated with DGS (1, 2). DGS is also found associated with other genetic abnormalities and with certain teratogenic influences. It was also recognized that multiple other clinical features could be associated with this deletion. The DGS phenotype is very heterogenous with variable expression of the different features including the immunodeficiency.

CAUSES OF DGS

EARLY THYMIC DEVELOPMENT

At an early stage of embryonic development the pharyngeal apparatus can be recognized. This becomes segmented into a series of pharyngeal arches and pouches each comprising an outer ectodermal and inner endodermal layer separated by mesodermal tissue and neural crest cells (NCC) (3, 4). The thymus, parathyroid glands and great vessels of the heart develop from these structures notably the third and fourth arch structures. Thymic epithelial development is under the control of the transcription factor, FoxN1, and studies of expression of this factor have demonstrated that the thymus derives from an area of the endoderm in the ventral aspect of the third pouch (5). The mesoderm and NCC contribute to the thymic connective tissue including vascular endothelium and mesenchymal cells, the latter thought to be important in regulating early thymic epithelial development (6). Parathyroid gland development is closely allied, this organ being derived from the endoderm of the ventral part of the third pharyngeal pouch again with mesodermal cells and NCC contributing the connective tissue and vascular endothelium. From the eighth week of human gestation, bone marrow derived T-cell precursors have been shown to enter the thymic structure (7). The further development of the thymus is dependent on two-way interactions between these lymphoid cells and the thymic stroma (8, 9).

Hematopoietic cell defects resulting in severe combined immunodeficiencies lead to failure or disturbed thymic development as a consequence of failure of this lymphoid – stromal interaction (10, 11) which can be reversed by successful hematopoietic stem cell transplantation (12). These aspects of thymic stromal deficiency are considered elsewhere in this Research Topic.

The classical features of DGS occur as a result of the early embryonic disturbance of development of the pharyngeal arch apparatus and are independent of the influence of hematopoietic cell precursors on thymic development.

GENETIC ASSOCIATIONS OF DGS

DiGeorge syndrome overlaps considerably with velocardiofacial (VCF) syndrome and to a lesser extent with conotruncal anomaly face syndrome; all these are associated with hemizygous 22q.11 deletions manifesting with a wide array of clinical features (13). The deletion is also associated with neurodevelopmental delay, behavioral, and psychiatric features. The multitude of possible clinical features (over 180) have been reviewed by Shprintzen (14). DGS and VCF are sometimes collectively referred to as the 22q.11 deletion syndrome. The incidence of this deletion is high at around 1:4000 (15). In 90–95% of cases this arises *de novo* with the other 5–10% being inherited from an affected parent (13). Over 90% of cases have a typical 3 Mb deletion including over 30 different genes (16). This seems to occur between two regions with homologous low copy repeats suggesting that deletion occurs through a process of homologous recombination. Most other patients have a smaller, 1.5 Mb, deletion (17, 18). There is no correlation between the size of the deletion and the clinical phenotype. Discordance between phenotypes has been described in monozygotic twins carrying the deletion (19). In rare cases mutations in a single gene, TBX1, have

been described resulting in the DGS phenotype (20, 21). TBX1 is one of the T-box genes with an important role in regulating the expression of transcription factors (22). Studies of a mouse model with a syngenic deletion on chromosome 16 have helped elucidate the role of Tbx1. Homozygous deletions of this gene result in a very severe, lethal phenotype including all the features of DGS whilst hemizygous loss of the gene produces a milder phenotype with variable penetrance of the different clinical features (23). However, implicating TBX1 as the sole gene causing DGS in 22q deletion syndromes may not be the whole story. Adjacent deletions not involving TBX1 can give a phenotype with some overlapping features (24) as can atypical deletions covering different critical regions in the same part of the chromosome (25). Other genes in the region, also affected in the typical DGS deletion, may have a modifying effect on expression of the disorder. These include CRKL, coding for an adaptor protein involved in growth factor signaling. Crkl is expressed in neural crest derived tissues and in mice null for the gene there is aberrant or absent thymic development (26). However, hemizygous Crkl loss is not associated with an abnormal clinical phenotype suggesting a gene dosing effect. The effect of compound heterozygosity for Tbx1 and Crkl deletions, on development of DGS features, is additive (27). The function of TBX1 is complex and mediated through regulation of downstream transcription factors. The detailed role of TBX1 in 22q.11 deletion syndromes and in thymus development in particular has been reviewed by others (28, 29).

A much rarer but well characterized genetic association with a DGS phenotype occurs with interstitial deletions at chromosome 10p (30–33). This has been designated DGS 2.The clinical phenotype overlaps with that associated with 22q.11 deletion but with some important differences. Sensorineural hearing loss and mental retardation are relatively common features in those with 10p deletions but rare in 22q11 deletion cases; renal anomalies, and general growth retardation are more prevalent in 10p deletion than in 22q11 deletion cases (34). Deletions at 10p syndrome have been estimated as having an incidence of 1 in 200,000, some 50 times less common than 22q.11 deletions (35, 36). The role of the genes deleted and responsible for the clinical picture is less well understood than in 22q deletion DGS but on-going work has identified some critical regions involved in developmental abnormalities (32, 37).

Mutations in the Chromodomain Helicase DNA-binding protein 7 (CHD7) gene are responsible for most cases of Colobomata, Heart defect, Atresia choanae, Retarded growth and development, Genital hypoplasia, Ear anomalies/deafness (CHARGE) syndrome. A DGS phenotype including complete athymia may be part of this syndrome but there is marked variability in expression of the multiple clinical features. The incidence has been estimated at 1 in 8500 (38). CHD7 acts as a regulator of transcription of other genes. Its expression has been demonstrated in the NCC of the pharyngeal arches. Normal development of these structures has been shown to be dependent on the co-expression of Chd7 and Tbx1 in mice suggesting the likely mechanism by which CHARGE syndrome can lead to a DGS phenotype (39, 40).

NON-GENETIC ASSOCIATIONS OF DGS

Embryopathy induced by exposure of the fetus to retinoic acid can include a DGS phenotype (41). Retinoic acid affects Tbx1 expression in avian embryos (42) whilst it has also been shown that Tbx1 can, in at least some circumstances, regulate retinoic acid metabolism (43). Fetal alcohol syndrome (44–46) and maternal diabetes (47, 48) have also been associated with the DGS phenotype. In the latter, there is often an associated renal agenesis. It has been postulated that maternal diabetes can lead to interference with neural crest and mesenchymal cell migration (49).

IMMUNOLOGICAL FEATURES OF DGS

INCIDENCE AND SEVERITY

DiGeorge syndrome may be associated with a complete range of T-cell deficiency from normal T-cell numbers and function to complete DGS (cDGS) with a T-negative severe combined immunodeficiency (SCID)-like picture. It was recognized early on that the T-cell immunodeficiency may be incomplete and the term partial DGS (pDGS) was coined (50). In a large series of patients with 22q11 deletions, the proportion of affected individuals falling into the cDGS category was around 1.5% of the 218 who underwent immunological testing or around 0.5% of the whole series of over 550 patients (13). A much higher proportion had minor laboratory abnormalities suggesting pDGS. In one series, from a major referral center, mild-moderate lymphopenia, consistent with pDGS, was reported in 30% of 22q.11 patients (51).

Less is known of the frequency of severe immunodeficiency in 10p deletion DGS. A review of published cases identified low levels of T cells and immunoglobulins as well as a small or hypoplastic thymus in 9 of 32 (28%) patients evaluated. However none of these patients were reported as having significant infections, suggesting that the immunodeficiency was likely partial rather than complete (34).

In CHARGE syndrome, severe immunodeficiency has been described (51–55). The proportion of cases affected with immunodeficiency is not well established as there is no reported large series looking at immunological parameters. Immunodeficiency may not always be considered in CHARGE; one recent report of a large series of 280 cases did not provide any information on the prevalence of recurrent infections or immunodeficiency (56). In a series of 25 cases (51), 16 (60%) were found to have lymphopenia. Only nine had full immunophenotyping performed and two of these had a picture of cDGS. A further five of eight patients dying in infancy had marked lymphopenia but did not have lymphocyte phenotyping performed so it is possible that the incidence of cDGS was higher. The authors do however concede that this series of patients referred to a specialist center might present a biased view. Nevertheless, the proportion of children with CHARGE syndrome affected by a significant immunodeficiency is probably at least as high as the proportion in DGS associated with 22q deletion. This conclusion would be consistent with the report of a series of 54 cases of patients referred for thymus transplantation for cDGS where the numbers of CHARGE and of 22q deleted cases were roughly in proportion to the incidences of the two genetic defects (55).

IMMUNODEFICIENCY IN PARTIAL DGS

The majority of children with thymic insufficiency as part of DGS, whatever the underlying cause, will have only a partial form of immunodeficiency. The consequences are an increased susceptibility to infections and sometimes immunodysregulation

resulting in autoimmunity. A wide range of T-cell immunity is seen in pDGS from near normal to near completely deficient. Normal or near normal T-cell numbers can be found even in those with an apparently absent or hypoplastic thymus and in these it is probable that some thymic tissue is ectopically placed (57). There may be a small subset of more severely deficient 22q.11 – pDGS patients with T-cell numbers near the lower end of the range who have an increased susceptibility to "T-cell" type pathogens such as *Candida albicans* and viral infections and an increased non-cardiac mortality (58, 59). Hypocalcemia was an associated feature of this subgroup in one of these studies (58) and was also associated with lymphopenia in another study of CHARGE patients (51). Otherwise there is no correlation between the severity of immunodeficiency and the clinical phenotype in regard the other features of DGS (60). Most pDGS patients do not suffer opportunistic or life-threatening infections. Their infections tend to be of a sinopulmonary nature, more consistent with a humoral than a T-cell immunodeficiency. Susceptibly to such respiratory tract infections is likely to be at least partly due to non-immunological issues such as velo-pharyngeal insufficiency, eustachian tube dysfunction, disco-ordinate swallowing, gastro-esophageal reflux, and sometimes tracheo-bronchomalacia (59, 61).

As is the case with other partial T-cell deficient states, autoimmune disease can occur in pDGS. This has most commonly been reported as manifesting with immune cytopenias, arthritis, or hyper/hypothyroidism (62–73). The mechanism by which tolerance breaks down leading to autoimmunity in pDGS is not clear. Many forms of primary immunodeficiency are associated with an increased risk of autoimmune disease including conditions not associated with dysregulation of T cells. It has been suggested that persistent antigen stimulation from frequent and/or persistent infections may predispose to autoimmunity (74). However, in pDGS autoimmunity is not predominantly found in those with the most severe or frequent infections (65, 75). It is more likely that disturbance of central or peripheral tolerance or both occur as a consequence of the thymic abnormality. In the normal situation, central tolerance is generated through the presentation of tissue specific peptides to developing thymocytes by medullary thymic epithelial cells in the context of autologous major histocompatibility antigens and under the regulation of the autoimmune regulator (AIRE). There is subsequent deletion (negative selection) of thymocytes recognizing these self-antigens. It is possible that a reduced bulk of thymic tissue in pDGS results in incomplete negative selection or that AIRE expression in pDGS is reduced or otherwise abnormal. The author is not aware of any reported studies of AIRE expression in thymic tissue from pDGS cases. Abnormalities of thymic tissue, including AIRE expression, has been described in SCID due to recombination activating gene (RAG) defects and may contribute to the multisystem inflammation/autoimmunity seen in Omenn syndrome (76) though these patients also have a defect of regulatory T cells suggesting a possible peripheral tolerance defect in addition (77). In pDGS, negative selection must occur in relation to most antigens since the autoimmune disease seen is usually limited to one or two organs or systems. By contrast, in autoimmune polyglandular syndrome type 1 (APS-1) (78) caused by mutations in the AIRE gene, multiple autoimmune disorders are typical. Breakdown of peripheral tolerance is another possible explanation for autoimmunity in pDGS. One study reported reduced numbers of circulating CD4+ Foxp3+ T cells, described as natural T regulatory cells (nTregs) in pDGS patients compared to controls. The levels of these cells correlated closely with the numbers of recent thymic emigrant cells suggesting they were at least partially thymus derived (75). Another study (79) looked at CD4+ CD25+ cells which include Treg cells. In both studies these populations were present in reduced numbers in pDGS patients compared to controls at all ages but there was no difference between the levels in patients with and without autoimmunity. Immunological assessment of pDGS patients often shows low overall numbers of T cells compared to normal with a tendency to improve after the first year of life, although in 10p deletion syndrome a progressive T-cell lymphopenia has been reported (33). Mitogen responsiveness is generally normal in pDGS (80, 81). An increase in T-cell numbers with age may in part be due to the development of oligoclonal expansions resulting in abnormal T-cell receptor spectratypes. (75, 82–85). Naïve T-cell proportions are lower than normal and fall off more quickly with age than in an age – matched control group (82). T-cell recombination excision circles (TRECs) were found to correlate well with the proportions of circulating naïve T cells (86), though a cautionary note was struck by the report of a patient, with what turned out to be pDGS, showing very low TREC levels with good naïve cell proportions (87).

Humoral immune defects and disturbance of B-cell immunity were recognized very early on after DGS was first described (50). These may be relevant to the types of infections suffered. A number of relatively small series have looked at immunoglobulin and antibody levels in DGS associated with 22q.11 deletion (62, 63, 65, 68, 75, 88–90) and CHARGE syndrome (51). Low immunoglobulin levels were reported with variable frequency, most commonly affecting IgM but also occasionally causing a sufficiently low IgG to merit immunoglobulin replacement therapy. Defective antibody responses to polysaccharide antigens were reported in a significant minority of patients. A recently published, much larger study reported on over 1000 patients, with a median age of 3 years, from the European Society for Immunodeficiency and US Immunodeficiency Network (91). Forty two percent were recorded as having 22q.11 deletion but the underlying cause was not reported in the remainder. Overall, 2.7% were on immunoglobulin replacement therapy (3% in those over 3 years old). In the over 3 years age group 6.2% had IgG levels below 5 g/l. Amongst patients over 3 years of age, around 0.7% had complete and 1% partial IgA deficiency whilst 23% had low levels of IgM. There was no association between low immunoglobulin levels, in any of the isotypes, and T-cell counts nor between low T-cell counts and immunoglobulin levels. The authors acknowledged that the data were incomplete and that there may have been some reporting bias in that these patients were registered through immunodeficiency networks. Nevertheless, this study provides the best estimate of the prevalence of humoral immune deficit in DGS. B-cell numbers were not reported in this study but in another study were found to be generally normal though sometimes low in the first year of life, normalizing later (92). The repertoire of IgH usage is also normal but further diversification through somatic hypermutation is

deficient (93). It has also been shown that the maturation of B-cells toward a memory phenotype is impaired in pDGS (88). Given the specific role of the thymus in T- but not B-cell development it is probable, but not proven, that B-cell abnormalities are secondary to the T-cell deficiency in these patients.

IMMUNODEFICIENCY IN COMPLETE DGS

Complete DGS is associated with athymia and results in a picture of SCID in a patient showing other variable features of DGS. Affected patients suffer opportunistic infections and, like other infants with SCID, are likely to die early unless they can be treated with a corrective procedure. In addition to susceptibility to infections these patients are at risk from transfusion acquired graft versus host disease (55).

In the typical form of cDGS the T-cell numbers are <50/cumm and mitogen responses are absent. B cells are usually present in normal numbers and NK cells in normal or high numbers. In a proportion of cases there may be some mature T cells present either through maternal engraftment (94) or through oligoclonal expansion of memory phenotype T cells which have developed without thymic processing (95). In the latter case, as in SCID these cells can mediate severe inflammation leading to an Omenn-like picture with erythrodermic rashes, enteropathy, and lymphadenopathy (53, 96) This is called atypical cDGS. The diagnosis of complete athymia then depends on showing absence (<50/cumm) of T cells with a naïve (CD3 + CD45 RA+CD62L+) phenotype as well as abnormal T-cell receptor usage either by T-cell receptor spectratyping or FACS analysis of usage of V Beta TCR chains (96). An example of the abnormal spectratype in an atypical cDGS patient is shown in **Figure 1** which can be compared to the normal spectratype achieved in the same patient after successful thymus transplantation (**Figure 2**). Mitogen responsiveness is usually, but not invariably, impaired in these atypical patients (96).

Diagnosis of cDGS depends on the findings of the clinical features of DGS together with the above immunological findings with or without identification of one of the associated genetic abnormalities. A recent report (97) describes two patients with absent T cells and DGS associated with 22q.11 deletion who were also found to have pathogenic mutations in the DCLRE1C (Artemis) gene, a classical cause of SCID. A clue to the latter diagnosis was the virtual absence of B cells as well as T cells which is very unusual in cDGS alone.

Newborn screening for SCID using TREC detection on blood spots has been in place in certain states of USA for around 3 years (98, 99). Since TRECs will be absent or extremely low (86) this allows the early diagnosis of cDGS. In the California program (98) screening of nearly one million newborns picked up one cDGS case who went on to thymus transplantation, eight with T-cell lymphopenia associated with 22q.11 deletion and one with CHARGE association. Picking up the latter group was useful in the early identification of these children as having significant immunodeficiency and allowed infection prevention measures to be put in place including avoidance of live viral vaccinations. Newborn screening programs should offer the opportunity of a better outcome through earlier intervention in both cDGS and some cases of pDGS.

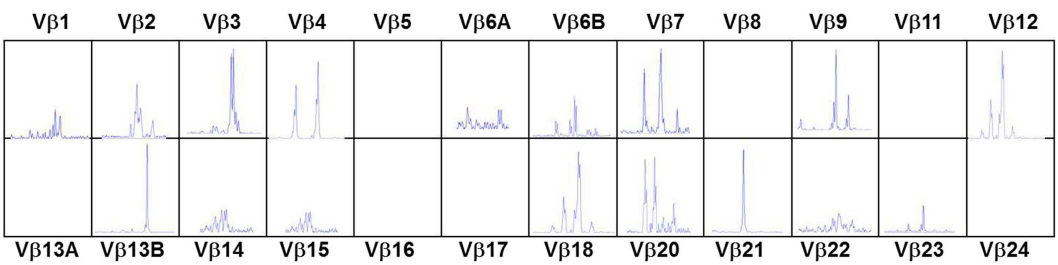

FIGURE 1 | T-cell receptor spectratyping of 24 Vβ families obtained using polymerase chain reaction amplification across the VDJ region and then plotting according to the size of the PCR products. Patient with atypical cDGS showing very abnormal spectratype with several completely missing families and abnormal skewed distribution in other families.

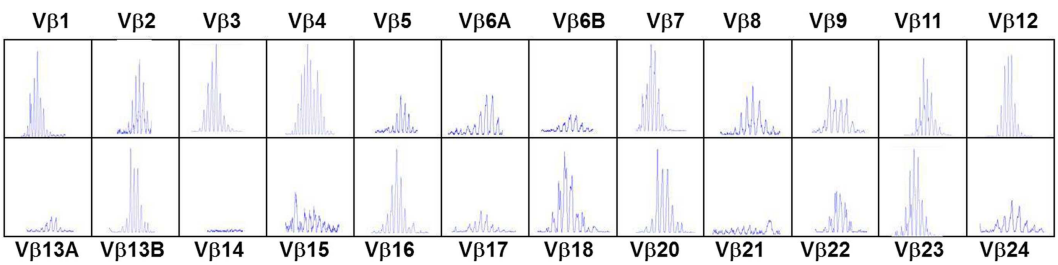

FIGURE 2 | T-cell receptor spectratyping performed as in legend to Figure 1. Same patient as in Figure 1, 23 months after thymus transplantation. Much more normal spectratype. All families represented mostly with Gaussian distribution.

CORRECTIVE TREATMENT FOR cDGS

HEMATOPOIETIC CELL TRANSPLANTATION

Treatment with hematopoietic cell transplantation (HCT) for athymia is dependent on the transfer of mature post-thymic T cells. Long term survival after such transplants has been reported (100, 101) though at a low rate (41–48%) compared to survival after HCT for SCID (102). Survival in the subgroup receiving matched sibling donor transplants was better at over 60% (100). Mortality was related to other features of DGS, to graft versus host disease and to pre-existing viral infections. The quality of immune reconstitution achieved, as expected, is poor with no evidence of naïve T cells and often low CD4 counts with skewed distribution of T-cell receptor usage. However immunoglobulin production and antibody responses were relatively good. Though overall the outcome after HCT for cDGS is not good, in some circumstances, such as overwhelming viral infection, HCT from a matched sibling may be life-saving (103).

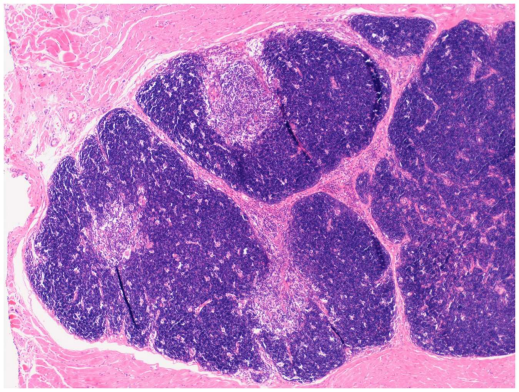

FIGURE 3 | Low-power view of a biopsy of transplanted thymus stained with Hematoxylin and Eosin. Normal looking thymic tissue surrounded by striated muscle. There is good corticomedullary distinction.

THYMUS TRANSPLANTATION

Replacement of thymic function using allografted tissue was first achieved using human fetal thymic tissue (104, 105). The use of post natal human thymus, necessarily removed at the time of cardiac surgery in infants undergoing median sternotomy, was pioneered by Markert at Duke University (106, 107) and has become established as the treatment of choice for cDGS. More recently this approach has also been used in London using an almost identical approach (manuscript in preparation). The thymus is cultured for 12–21 days prior to transplantation into the quadriceps muscle of the patient. During this period most thymocytes are washed out or undergo apoptosis whilst the thymic stroma is preserved. Patients with atypical cDGS are pre-treated with anti thymocyte globulin and continuing cyclosporine A (108) whilst typical cases receive no pre-conditioning. The results have been published (55, 109) and of 60 patients treated 43 survived (72%). This compares favorably with the outcome after HCT described above though strict comparison is not possible as the thymus transplant patients were a selected group. After successful transplantation, patients develop host derived naïve T cells with a normal T-cell receptor repertoire (**Figure 2**), normal mitogen responses and antigen specific immune responses restricted to the host major histocompatibility complex (MHC). There is normalization of the TCR repertoire in circulating regulatory T cells (110). Biopsies of transplanted thymus taken from 2 months onward show thymopoiesis (111) and normal thymus architecture (**Figure 3**). The levels of circulating T cells achieved do not usually match normal age matched controls and are more akin to the levels seen in children with pDGS. Tolerance to the donor's MHC has been demonstrated (112) and this has been exploited to enable parathyroid transplantation from a parent in situations where there is coincidental partial MHC class 2 matching between the donor and the parent (113).

Deaths after thymus transplantation were related mainly to pre-existing co-morbidities, mostly chronic lung disease and systemic viral infections such as cytomegalovirus (CMV) (114). This virus is a particular problem. Screening of potential thymic donors always excludes CMV positive donors but a proportion of cDGS patients will have acquired the virus before thymus transplantation. Biopsies of transplanted thymus tissue from two patients with CMV in the Markert series showed no evidence of thymopoiesis even though the epithelium was viable (111). Both patients died. A similar appearance was found in a CMV infected patient in London who also died without evidence of thymopoiesis (manuscript in preparation). The mechanism by which CMV interferes with thymopoiesis is not clear but as a result of this experience, CMV infection should be considered at least a relative contraindication to thymus transplantation. After successful thymus transplantation patients are able to control infections and to come off antibiotic prophylaxis and immunoglobulin therapy with normal responses to immunization. The main problem that has been encountered is the development of autoimmunity. Around one third of patients have shown autoimmunity, mainly hypothyroidism but also with a significant number of immune cytopenias (109). It is interesting that this spectrum of autoimmunity is similar to that seen in pDGS patients, as discussed above, and may have the same causation or may be related to faulty thymic education related to the fact that the transplanted thymic epithelial cells are not MHC matched, as discussed below. No clinical or methodological correlates with risk of autoimmune development could be identified in the Duke University series. (114).

The success of transplantation of thymus which is not matched at the MHC loci offers interesting insights into thymocyte development. In particular, it suggests that positive and negative selection of developing thymocytes can occur in the absence of self MHC expressed on thymic epithelial cells. The mechanism by which this takes place is incompletely understood. Reconstitution experiments in nude mice with MHC incompatible thymic tissue showed that functional T cell development could be supported by haematopoeitic cell-expressed MHC instead of TEC-expressed MHC (115). Further work showed that development of functional CD4 (but not CD8) cells however does seem to require interaction with MHC on TECs but not any particular allelic form of MHC (116). Under the influence of AIRE expressed on thymic epithelium dendritic cells have been shown to have a role in negative selection in mice (117). Whilst negative selection may be imperfect resulting in autoimmunity in some cases, it must be

largely effective since multiple system/organ autoimmunity from widespread lack of central tolerance has not been seen. Positive selection has also been shown to be mediated by fibroblasts (118) and by thymocytes (119, 120). Influx of these cell types expressing host MHC to the developing thymus allograft could therefore have the potential for mediating the selection processes.

CONCLUSION

Study of the thymic deficiency in DGS provides insights into the development of the thymus and the mechanisms of thymopoiesis required to generate a robust and diverse T-cell mediated immunity. Thymus transplantation offers a novel way of correcting the immunodeficiency in this disorder.

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Lymphopenia-induced proliferation in Aire-deficient mice helps to explain their autoimmunity and differences from human patients

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Studies on autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and its mouse model – both caused by mutant *AIRE* – have greatly advanced the understanding of thymic processes that generate a self-tolerant T-cell repertoire. Much is now known about the molecular mechanisms by which AIRE induces tissue-specific antigen expression in thymic epithelium, and how this leads to negative selection of auto-reactive thymocytes. However, we still do not understand the processes that lead to the activation of any infrequent naïve auto-reactive T-cells exported by AIRE-deficient thymi. Also, the striking phenotypic differences between APECED and its mouse models have puzzled researchers for years. The aim of this review is to suggest explanations for some of these unanswered questions, based on a fresh view of published experiments. We review evidence that auto-reactive T-cells can be activated by the prolonged neonatal lymphopenia that naturally develops in young Aire-deficient mice due to delayed export of mature thymocytes. Lymphopenia-induced proliferation (LIP) helps to fill the empty space; by favoring auto-reactive T-cells, it also leads to lymphocyte infiltration in the same tissues as in day 3 thymectomized animals. The LIP becomes uncontrolled when loss of Aire is combined with defects in genes responsible for anergy induction and Treg responsiveness, or in signaling from the T-cell receptor and homeostatic cytokines. In APECED patients, LIP is much less likely to be involved in activation of naïve auto-reactive T-cells, as humans are born with a more mature immune system than in neonatal mice. We suggest that human AIRE-deficiency presents with different phenotypes because of additional precipitating factors that compound the defective negative selection of potentially autoaggressive tissue-specific thymocytes.

Keywords: AIRE, APECED, lymphopenia-induced proliferation, thymus, negative selection, autoantigens, immune privilege, NOD

INTRODUCTION

The autoimmune regulator (AIRE) is a transcriptional activator with a restricted expression pattern and important functions in medullary thymic epithelial cells (mTECs) (1). The thymus is the organ where a self-tolerant T-cell repertoire is established via positive and negative selection of thymocytes. To ensure tolerance toward the set of tissue-specific antigens (TSAs) from different peripheral organs, mTECs "promiscuously" express thousands of TSAs that are then presented to developing thymocytes; one of the best known among them is insulin (2, 3). AIRE is the best characterized transcriptional regulator in mTECs. It is generally accepted that its main thymic role is to ensure negative selection of thymocytes with T-cell receptors (TCRs) with high affinities for epitopes from TSAs. At first sight, this idea seems to fit with the variety of endocrine, ectodermal, and lymphoid autoimmune diseases that present in patients with *AIRE* mutations and comprise the Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) or autoimmune polyendocrine syndrome type I (APS-I) syndrome (4–6). However, there is curiously little discussion about how these infrequent naïve auto-reactive T-cells that escape negative selection in AIRE-deficient thymi are activated to cause disease in the periphery, or about the rather consistent early onset of its highly unusual cardinal manifestations, or about the strikingly different phenotypes in $Aire^{-/-}$ mice (7–9). **Table 1** lists the autoimmune features of AIRE-deficient humans vs. mice and highlights their surprisingly limited overlap (7–21). Here, we propose the hypotheses that defective thymic negative selection is not sufficient by itself to induce autoimmunity and that these differences in disease phenotypes reflect distinct varieties of additional influences in $Aire^{-/-}$ mice vs. humans.

AIRE IS RESPONSIBLE FOR NEGATIVE SELECTION OF TSA-SPECIFIC THYMOCYTES

The normal roles of Aire in TSA up-regulation by mTECs, and thus in central tolerance induction, are firmly established. In mice transgenic for single TCRs specific for immune-dominant epitopes from hen egg lysozyme (HEL) or ovalbumin (OVA), large proportions of thymocytes are efficiently deleted if their neo-self-antigens are expressed under Aire-dependent gene promoters. Membrane-bound HEL or OVA (mHEL or mOVA) under the rat

Table 1 | Phenotypes and autoantibodies differ between APECED patients and *Aire*$^{-/-}$ mice.

APECED patients[a]	*Aire*$^{-/-}$ mice[b]	APECED patients[a]	*Aire*$^{-/-}$ mice[b]
DISEASES/IMMUNE CELL INFILTRATIONS		**AUTOANTIBODIES TO:**	
Chronic mucocutaneous candidiasis		Type I IFNs	
Hypoparathyroidism		IL-22, IL-17F, **IL-17A**	**IL-17A** (IL-17F) (11)
Addison's disease		NALP5	
Ovarian failure	**Infertility**	CaSR	
Testicular failure		P450c17, P450c21, P450scc	
Hypopituitarism		IA-2, GAD65	
Autoimmune hepatitis	Liver infiltration	TG, TPO	
Intestinal dysfunction		TDRD6	
Pancreatitis		AADC	
Tubulointerstitial nephritis		P450 1A2	
Interstitial lung disease	Lung infiltration	TPH	
Alopecia		HDC	
Vitiligo		TH	
Rash with fever		SOX9/SOX10	
Asplenia		KCNRG	
Keratoconjunctivitis		Myelin protein zero (12)	
Dental enamel dysplasia		LPLUNC1 (13)	Vomeromodulin (13)
Nail dystrophy		**BPIFB1** (14)	**BPIFB9** (14)
Type 1 diabetes			OBP1a (16)
Hypothyroidism			SVS2 (17)
CIPD (10)			IRBP (15)
Pernicious anemia	Gastritis		alpha-fodrin (18)
	Uveoretinitis		TRP-1 (19)
	Dacryoadenitis		Mucin 6 (20)
	Salivary gland infiltration		

[a] *Autoimmune phenotypes of APECED patients and their autoantibody reactivities are summarized from (21).*

[b] *Summarized from (9), only Aire*$^{-/-}$ *mice on C57BL/6 and BALBc backgrounds without additional immune defects are included.*

CIDP, Chronic inflammatory demyelinating polyneuropathy; NALP5, NACHT leucine-rich-repeat protein 5; CaSR, calcium-sensing receptor; P450c17, steroid 17-α-hydroxylase; P450c21, steroid 21-hydroxylase; P450scc, side chain cleavage enzyme; IA-2, islet antigen-2; GAD65, glutamic acid decarboxylase; TG, thyroglobulin; TPO, thyroid peroxidase; TDRD6, tudor domain containing protein 6; AADC, aromatic l*-amino acid decarboxylase; P450 1A2, cytochrome P450 1A2; TPH, tryptophan hydroxylase; HDC, histidine decarboxylase; TH, tyrosine hydroxylase; KCNRG, potassium channel-regulating protein; BPIFB1, 1 bactericidal/permeability-increasing fold-containing B1; OBP1a, odorant binding protein 1a; SVS2, seminal vesicle secretory protein 2; IRBP, interphotoreceptor retinoid-binding protein; TRP-1, tyrosinase-related protein-1; LPLUNC1, Long palate lung nasal epithelium clone.*

Shared autoimmune features are indicated in bold.

insulin promoter (RIP) is expressed in both pancreatic β cells and the thymus (22, 23), and mHEL under the interphotoreceptor retinoid-binding protein (IRBP) promoter in both retina and thymus (24). When these mice are crossed with the respective TCR-transgenic animals, their clonotypic thymocytes are deleted with 75–97% efficiency, but only in mice with intact Aire, highlighting its indispensable role in negative selection. Moreover, the prevalence of neo-self-antigen-reactive T-cells is reduced still further in the periphery, underlining the importance of active peripheral tolerance mechanisms.

Interestingly, expression levels of the transgenes in the thymus varied in different studies. In a retinal neo-self-antigen model, the transgenic mRNA (*Escherichia coli* β-galactosidase under arrestin promoter) was undetectable even in the wild-type (wt) thymus (25). Whereas mHEL showed the expected Aire-dependent pattern of higher expression in wt than *Aire*$^{-/-}$ mTECs (24, 26) (when driven by the insulin or IRBP promoters), transcript levels for RIP-driven mOVA were not markedly decreased in *Aire*$^{-/-}$ thymi (22). This raises the possibility that, besides up-regulation of TSAs in the thymus Aire plays additional roles in generating self-tolerance, e.g., inducing the maturation of mTECs, as reviewed recently (27, 28). Loss of Aire also alters thymic architecture and mTEC ultrastructure (29, 30), and these effects reach back even to the immature Aire-negative mTEC subset (31). Indeed, there are reports that Aire-deficiency leads to breakdown of tolerance even to apparently Aire-independent antigens (18). Moreover, the development of the most mature single CD4 positive thymocyte subpopulation (CD69^{-}, Qa-2^{+}) is impaired in Aire-deficient thymi (32).

The role of Aire in negative selection has also been studied in TCR-transgenic models where clonotypic T-cells are targeted

toward naturally expressed self-antigens such as the melanocyte-/melanoma-specific tyrosinase-related protein-1 (TRP-1). In these mice (on a $Rag^{-/-}$ background), negative selection again depended on Aire; when its only change was the dominant negative *Aire* G228W point mutation, melanoma growth was decreased. Surprisingly, however, vitiligo was not reported in this study, although TRP-1 is also expressed in normal melanocytes (19).

The role of Aire in negative selection has also been studied in another TCR-transgenic model with reactivity to the major retinal autoantigen – IRBP. Although its thymic expression is reportedly Aire-dependent, clonotypic thymocytes were not deleted in any of three transgenic mouse lines on the uveitis-susceptible B10.RIII background (33). On the contrary, in two of them, the majority of CD4 single positive thymic T-cells bound IRBP–MHC dimers; strikingly they were several-fold more frequent than in wt animals (33). Uveitis developed spontaneously in these two mouse lines, but not in the third, where frequencies were lowest in both thymus and periphery: 6 and 1% respectively; those were still much higher than in $Aire^{-/-}$ mice with no TCR-transgene (34). Clonotypic T-cell deletion was also incomplete in mice transgenic for an insulin B chain epitope-specific TCR, only a fraction of which developed diabetes (35).

Several studies have confirmed the importance of thymic negative selection of auto-reactive T-cells in physiological settings, i.e., in mice with un-manipulated T-cell repertoires (34, 36). Indeed, thymic stromal or lymphoid cells were necessary to confer tolerance to the central nervous system (CNS) antigen myelin proteolipid protein (PLP) (36). Importantly, susceptibility to experimental autoimmune encephalomyelitis (EAE) in SJL/J mice could be explained by the exclusion of the immunodominant epitope of PLP (for this strain) from the thymic isoform of PLP, and the export of potentially auto-reactive cells to the periphery (36). However, this model of EAE in SJL/J mice does not develop spontaneously, but requires immunization with antigen emulsified in complete Freund's adjuvant (CFA).

NAÏVE AUTO-REACTIVE T-CELLS DO NOT CAUSE AUTOIMMUNITY BY DEFAULT

According to current models, AIRE's main role is to ensure negative selection of TSA-specific thymocytes. If so, self-reactive T-cells escaping from $Aire^{-/-}$ thymi must normally be naïve and infrequent. Even when frequencies are much higher in TCR-transgenic models, disease penetrance is not always 100%, especially when the TCRs are expressed in CD4+ T-cells. In the TCR–TrpHEL model, with neoantigen expression in melanocytes, 12% of the animals remained free of vitiligo (37); in an RIP–OVA OTII model with neo-self-antigen expression in pancreatic β-cells, about 1/3 were persistently non-diabetic (23) in spite of large numbers of auto-reactive T-cells in the periphery. TSA-specific T-cells are much less frequent in $Aire^{-/-}$ animals with un-manipulated T-cell repertoires. How their uncommon naïve thymic emigrants are activated to induce autoimmune disease in the periphery remains unexplained, one might expect them to get tolerized instead (38, 39). Indeed, when naïve T-cells encounter self-antigen in tissue-draining lymph nodes or spleen in wt mice, they undergo an initial burst of proliferation that is followed by deletion and anergy (40–44) or acquisition of regulatory T-cell (Treg) phenotypes (35, 45). In intriguing contrast, autoimmunity readily develops when naïve auto-reactive T-cells are transferred to lymphopenic hosts (46, 47).

LYMPHOPENIA TRIGGERS AUTOIMMUNITY IN *AIRE*$^{-/-}$ MICE

The striking similarities in manifestations in $Aire^{-/-}$ and day 3 thymectomized mice (d3tx) have been noticed earlier (48–50). Both models show inflammatory infiltrates in similar tissues plus autoantibodies against some of their antigens in: stomach, thyroid, ovaries, prostate, pancreas, lacrimal and salivary glands, and testis (9, 18, 50–55). With both types of models, the manifestations even follow the same strain-specific preferences: e.g., generally lower autoimmune susceptibility in C57BL/6 mice, whereas gastritis is the most prevalent feature on the BALBc background.

In d3tx mice, the autoimmunity is explained by prolonged lymphopenia-induced proliferation (LIP) of auto-reactive lymphocytes that out-compete Tregs in susceptible animals (56, 57). Although normal neonatal mice show a physiologic lymphopenia, it does not induce substantial LIP (56). We have shown that, besides inducing TSA expression, thymic Aire normally upregulates several chemokines, especially CCR7 and CCR4 ligands, that attract immature thymocytes to the medulla. Their corticomedullary migration is delayed in $Aire^{-/-}$ mice, and that, in turn, delays the export of their mature progeny, prolonging the postnatal lymphopenia at least through day 5 (31). Interestingly, mice deficient in CCR7 (or its ligands) show not only similar delays in T-cell emigration from the thymus but also inflammatory infiltrates in the very organs listed above (58–60). We therefore hypothesize that LIP also contributes to these inflammatory infiltrates and compensates for the relatively low numbers of naïve auto-reactive T-cells that escape from $Aire^{-/-}$ thymi. This notion is supported by the evidence that the lymphopenia in irradiated $Aire^{-/-}$ mice increases the gastric autoimmunity (20); and that Aire expression is required only in the fetal and early post-natal periods to prevent autoimmunity (48).

Lymphopenia-induced proliferation is sometimes classified according to the rate of division of T-cells to homeostatic and spontaneous proliferation (56). It is highest when chronically lymphopenic adult mice are reconstituted with low numbers of lymphocytes (56, 61). In this case, T-cells respond to antigens derived from commensals, which probably translocate from the gut to lymphoid organs due to the host immunodeficiency (61). Commensals seem unlikely contributors to the LIP that occurs early in life, e.g., in d3tx mice. Nevertheless, LIP favors auto-reactive cells, as they get stronger signals through their TCRs as well as from homeostatic cytokines (IL-7 and IL-15) that are upregulated in lymphopenic hosts. As they concomitantly differentiate, these T-cells acquire the markers of activated memory cells ($CD44^{+}CD62L^{-}$) (62–66).

There are several indications of homeostatically proliferating T-cells in $Aire^{-/-}$ mice, including signs of oligoclonality (67). Whereas thymocytes from Aire-deficient and wt mice showed no differences in TCR Vβ-chain CDR3 length and spectratype, splenic T-cells from $Aire^{-/-}$ mice showed a clear alteration in the TCR repertoire distribution in 3 out of 24 Vβ families at 2 and 6 months of age (67). A more recent study also found slight perturbations in CDR3 Vβ length distribution, and significantly higher percentages of CD44+ T helper cells in spleens and lymph nodes of $Aire^{-/-}$

mice than in wt controls (9). CD44 up-regulation in T-cells from *Aire*$^{-/-}$ mice was also noted by Anderson et al. (68).

Looking for further activation of auto-reactive cells in lymphopenic conditions, Kekalainen et al. (69) transferred lymph node cells from *Aire*$^{+}$ and *Aire*$^{-/-}$ mice to immunodeficient hosts. However, although especially the CD8+ *Aire*$^{-/-}$ T-cells proliferated more, there was no clinical disease, and the mild infiltrates in the livers, salivary glands, and pancreata did not differ from those in the controls. The rare auto-reactive cells in these animals had probably already been tolerized by peripheral mechanisms in the donors themselves. This suggests that prolonged lymphopenia in the neonatal period, together with export of naïve cells to the periphery, contributes substantially (but not exclusively) to the development of inflammatory infiltrates in *Aire*$^{-/-}$ mice, and that the auto-reactive cells are subject to regulation in the periphery that prevents serious damage to the target organs.

Certain TCR-transgenic T-cells are also prone to homeostatic proliferation. These include the MHC-class I-restricted OT-I line recognizing a peptide from OVA (62). Interestingly, spontaneous diabetes already appears in neonatal RIP–OVA *Aire*$^{-/-}$ OT-I mice (22). This severe autoimmunity might well have been potentiated by perinatal activation of the transgenic T-cells in these lymphopenic hosts.

AIRE AND LIP IN AUTOIMMUNITY AGAINST PRIVILEGED ORGANS

Autoantigens from some organs like the CNS/retina were thought to be sequestered from the immune system, which might therefore not be fully tolerant to them. It has been suggested that AIRE might play especially important roles in protecting these organs from autoimmune attack, e.g., provoked by local infections (49). Indeed, central deletion of auto-reactive thymocytes would be a particular priority for CNS and eye antigens, as regeneration is minimal in these tissues, and their peripheral tolerizing mechanisms might be inefficient. The intraocular compartments are isolated from the circulation – by barriers formed by tight junctions between the endothelial cells of the ciliary blood vessels, and between the lining epithelial cells; also in the retinal pigment epithelium (RPE) and the local endothelium (70–72). These barriers are impermeable to circulating soluble macromolecules and most cell types except for activated T-cells and immature antigen-presenting cells (APCs). In the other direction, any soluble retinal antigens (such as IRBP) shed physiologically or injected experimentally can drain via the aqueous fluid and episcleral veins to reach the thymus, liver, and spleen (70). The resulting systemic tolerance is termed anterior chamber-associated immune deviation (ACAID). The presumed privilege of the eye used to be attributed to paucity of APCs and lymphatics, but it is now known that there are rich networks of APCs and a functioning lymphatic system draining all parts of the eye, except the retina proper, via the submandibular node (70–72). Thus, ocular privilege is not due to a passive barrier, but instead depends on inducible active processes that can be transferred by immune cells.

One prominent feature in *Aire*$^{-/-}$ mice is their retinal disease. Although it is extremely rare in APECED patients who frequently suffer from keratito conjunctivitis (4, 73), it affects ~30% of these mice by age 20 weeks on a C57BL/6 background (34). Recently, they were backcrossed onto the autoimmune uveitis-susceptible B10.RIII background to monitor eye pathology more carefully (74). Surprisingly, the spontaneous disease was milder on the *Aire*$^{-/-}$ background than in the other two models (induced by immunization with IRBP + CFA or arising spontaneously in IRBP TCR-transgenic mice), and rarely caused blindness. Instead, it presented with relatively low-grade but multi-focal retinal inflammation and severe choroiditis, possibly hinting at moderately potent regulatory mechanisms.

There are many indications that EAU is enhanced by LIP of self-reactive T-cells (33, 75, 76). In intact wt recipients, IRBP-transgenic T-cells only induced uveitis after antigen-activation: recipients of naïve cells, even from the highest transgenic TCR-expressing line, remained disease-free. In telling contrast, naïve T-cells did induce disease when transferred to lymphopenic *Rag2*$^{-/-}$ recipients, again implicating LIP in converting them into effector cells (33). In the same study, LIP was evidenced in the mouse lines with higher prevalences of TCR-transgenic T-cells by increases in CD44^{+}CD62L^{-} activated T-cells, even in peripheral lymph nodes that do not drain the eye. This implicates LIP in these transgenic animals too, possibly due to aberrant thymic development, and probably lymphopenic periods earlier in life (33). LIP has also been identified as a potent activator of EAU in another transgenic model (76) and, interestingly, uveoretinitis develops in unimmunized d3tx mice if subsequently injected with anti-CD25 to deplete CD25^{+}CD4^{+} Tregs (75).

REVERSAL OF LYMPHOPENIA ALLEVIATES AUTOIMMUNITY

Autoimmunity that results from LIP should be down-modulated by transfer of lymphocytes. This indeed occurs in *Aire*$^{-/-}$ mice, where the appearance of inflammatory infiltrates could be suppressed by introducing a controlled excess of T-cells from normal donors – by co-transplanting 1:4 mixes either of *Aire*$^{-/-}$: wt stroma from thymic lobes, or of splenocytes, into athymic or *Rag*$^{-/-}$ recipients, respectively (22).

As the phenotypes of *Aire*$^{-/-}$ mice are so mild, it is difficult to dissect the mechanisms that might be modulating their autoimmunity. Therefore, crosses of *Aire*$^{-/-}$ with NOD mice have been used, as they develop earlier and more severe autoimmunity (48). In these crosses, Aire expression is especially important during perinatal life. Moreover, intraperitoneal injection of adult T-cells on days 1 and 7 conferred significant but not complete protection from this exaggerated autoimmunity (48) (see below).

IS ABSENCE OF SELF-ANTIGEN FROM THE THYMUS SUFFICIENT BY ITSELF TO INDUCE ORGAN-SPECIFIC AUTOIMMUNE DISEASE?

It is sometimes assumed that the autoimmunity results solely from the absence of a single autoantigen from the thymus in the presence of wt Aire. That is apparently contradicted by our hypothesis that prolonged lymphopenia in *Aire*$^{-/-}$ mice is an important cofactor for auto-aggression, so we now discuss two models that might help to distinguish between these possibilities.

DeVoss et al. identified IRBP as the major target in autoimmune uveitis in *Aire*$^{-/-}$ mice (15). Its thymic expression is Aire-dependent, although it is barely detectable in wt thymic stroma. Absence of IRBP in the thymic compartment alone was sufficient

to cause disease when athymic nude mice were transplanted with fetal thymic stroma from IRBP$^{-/-}$ mice or wt mice. Mononuclear infiltrates appeared in their retinae, but not in recipients of wt stroma. Here again, lymphopenia must have been an important early contributor, as the first thymic emigrants appeared to abnormal lymphopenic adults.

When DeVoss et al. also crossed *Aire*$^{-/-}$ with IRBP$^{-/-}$ mice, the retinae showed no infiltrates, as expected because there was no target for the IRBP-specific cells to attack. However, IRBP is secreted, and even reaches the vitreous, and eventually drains to the spleen and lymph nodes (77). Hence this major eye retinal autoantigen was missing from the peripheral immune system too, and was not available to fuel homeostatic proliferation of IRBP-specific T-cells. Also the IRBP$^{-/-}$ retina is atrophic and might be depleted of other autoantigens.

Interestingly, when mice transgenic for mHEL under the IRBP promoter were crossed with HEL-specific TCR-transgenic mice, they showed severe spontaneous EAU even on a wt *Aire* background (24). Negative selection of clonotypic T-cells was not complete in this model, and many neo-self-antigen-specific T-cells were exported to the periphery. The mHEL – unlike soluble IRBP itself – may have failed to access lymphoid organs/induce peripheral tolerance. The resulting disease was already so severe that any exacerbating effect of Aire-deficiency was not detectable. If these HEL-specific clonotypic T-cells were susceptible to LIP due to cross-reactivity with some self epitopes (which has not been checked), that might well have contributed too.

In another study, mice were engineered specifically to prevent any insulin expression in mTECs, and to use only one of the two insulin genes (*Ins2*) in their pancreatic β-cells (78). They developed spontaneous diabetes within 3 weeks after birth. However, there are also some caveats with this study (79). The diabetes was not transferrable to immunodeficient adult hosts with lymphocytes or thymi from the transgenic mice, which showed only moderate insulitis (80). This apparently implicates the additionally impaired physiology of *Ins1*$^{-/-}$ β-cells (compensatory hyperplasia, increased death during the developmental wave of apoptosis that occurs in normal development) in disease initiation in very young mice (81). In this model again, loss of thymic negative selection alone was not sufficient to cause clinical disease. Furthermore, since insulin is already secreted in the fetus, it should normally be available for thymic deletion, e.g., when presented by medullary dendritic cells, without promiscuous expression in mTECs, but its levels may be decreased prenatally in *Ins1*$^{-/-}$ mice, reducing its availability for negative selection.

AIRE-DEFICIENCY BECOMES LETHAL IF PERIPHERAL BACK-UP MECHANISMS ARE ELIMINATED

Two highly informative crosses of *Aire*$^{-/-}$ mice – with strains with other immune defects – underline the importance of back-up mechanisms that are apparently responsible for the mildness of the disease phenotypes in *Aire*$^{-/-}$ mice. Crosses onto *Cbl-b*-deficient or diabetes-prone NOD backgrounds show astonishing similarities (39, 53, 82). They both suffer from early wasting disease and succumb to acute exocrine pancreatitis around 3–4 weeks of age. *Aire*$^{-/-}$/*Cbl-b*$^{-/-}$ mice showed additional lymphocytic infiltrates in submandibular salivary glands and stomach (39), while Aire-deficiency on the NOD background was accompanied by severe pulmonitis and infiltrates in liver, salivary gland, prostate, ovary, stomach, and thyroid (53, 82).

Interestingly, mice deficient in Cbl-b alone are healthy in the absence of additional triggers (83), so it was a major surprise that crossing with *Aire*$^{-/-}$ mice led to such severe disease. Cbl-b normally renders naïve T-cells highly dependent on co-stimulation; when it is deleted, they are "trigger-happy," and much less susceptible to anergy. Clonal deletion of CD8+ T-cells also depends on Cbl-b, and Cbl-b-deficient T-cells are partially resistant to Treg cell-mediated suppression (83). Furthermore, induction of Tregs from naïve precursors is likewise impaired in the absence of Cbl-b (84).

The CD44+ memory phenotype T-cells generated by LIP are normally restrained by Tregs that proliferate rapidly in d3tx mice and are crucial for preventing autoimmunity in lymphopenic animals (50, 85). In *Aire*$^{-/-}$/*Cbl-b*$^{-/-}$ mice, readier activation of homeostatically proliferating T-cells, impaired induction of peripheral Tregs and lower responsiveness of proliferating lymphocytes to the influence of Tregs are probably responsible for their severe early autoimmunity. The proportions of CD4+ and CD8+ T-cells with CD44^{high} were greatly increased in these double knock-outs. This supports the idea that LIP is participating during prolonged lymphopenia in *Aire*$^{-/-}$ mice, where "trigger-happy" polyclonal T-cells proliferate in response to available self-peptide-MHC complexes in the presence of homeostatic cytokines.

Interestingly, the immune defects in NOD mice include mild lymphopenia and dysregulated function of homeostatic cytokines (46). Indeed, T-cell transfer and CFA injection protect NOD mice against diabetes (46). The efficiency of their thymic selection has been a matter of controversy; recent data are in line with normal negative selection but impaired positive selection in NOD mice due to selective defects in the Erk1/2 signaling module downstream of TCR (86) that is important for T-cell survival and tuning of TCR responsiveness. In the periphery, anergy induction appears normal in NOD T-cells. Insulin-specific effector T-cells were generated in pancreatic lymph nodes only between 3 and 5 weeks of age, at the time of increased release of β-cell antigens (87). In all mouse strains, a wave of β-cell apoptosis occurs during the neonatal period, peaking at 9–15 days, but apoptotic debris is cleared less efficiently in NOD mice (88). Interestingly, diabetes is accelerated in mice thymectomized at week 3 – i.e., precisely when β-cell-specific T-cells are initially activated – when Tx caused moderate lymphopenia. Furthermore, the timing of that lymphopenia is evidently critical in target organ selection; while d3tx in NOD mice did not affect diabetes incidence, gastritis became much commoner (88). Indeed, this *Aire*$^{-/-}$/NOD combination may maximize homeostatic proliferation just when exocrine pancreatic antigen release is greatest. The combination of impaired positive selection in NOD mice with delayed migration of thymocytes into the Aire$^{-/-}$ medulla apparently amplifies the neonatal lymphopenia, which is further exaggerated by hyper-responsiveness of NOD T-cells to IL-21 and poor T-cell survival. Homeostatically proliferating cells compete for IL-7 and/or available MHC/(cross-reactive) self peptides (56). Therefore the absence of diabetes in *Aire*$^{-/-}$/NOD mice may implicate the early

proliferation of T-cells that encounter other available autoantigens and fill the space before the β-cell antigens are released.

Why the autoimmune attack focuses on the exocrine pancreas remains obscure. We suggest that three peculiarities of neonatal mice might be relevant: (1) readier access of neonatal T-cells to peripheral organs (89) where they normally differentiate into TSA-specific Tregs (45). Interestingly, this conversion to Tregs is subverted by IL-7 (45); (2) rapid changes and increased blood flow to certain organs (lungs, pancreas, liver, and intestine) after birth that renders their antigens more accessible to T-cells; (3) autophagy that is naturally upregulated immediately after birth – to adapt to the loss of the constant trans-placental supply of nutrients – especially in muscle/diaphragm, heart and lungs; also the pancreas, which undergoes major changes after birth too, to meet the demands for the proteolytic enzymes it must now secrete (90). Their premature intracellular activation in autophagolysosomes, together with autoimmune attack by "trigger-happy" homeostatically proliferating T-cells, might greatly exacerbate the tissue damage. The thymic involution in *Aire*$^{-/-}$/*Cbl-b*$^{-/-}$ mice could be the result of stress or a "cytokine storm" created by this fulminant pancreatic disease.

TREG CELLS IN AIRE-DEFICIENCY

Studies in APECED patients have shown significantly lower Treg numbers and function than in healthy controls (91–94). Whether this is a direct effect of the thymic AIRE-deficiency or secondary to the severe autoimmune diseases in these patients remains unknown. By contrast, the role of Aire-deficiency in the development of Treg cells in the mouse thymus is controversial. Many studies have reported that their numbers are unchanged (9, 18, 26, 95), but others have found them reduced (22, 96, 97). In peripheral organs, their numbers and function are similar to those in wt mice (9, 22). Recently, Malchow et al. showed appearance of Tregs specific for an Aire-dependent TSA that proliferated in tumors and could therefore interfere in their rejection (96). The autoimmunity in d3tx mice was initially thought to arise because of significantly later maturation and release of Tregs than of effector cells (55). However, Tregs proliferate equally well in d3tx lymphopenic hosts, which is important in the prevention of autoimmunity (50, 64). Interestingly, LIP is even greater in Tregs from *Aire*$^{-/-}$ than wt mice when transferred to lymphopenic hosts (69).

One of the crosses that showed no additive effect on the phenotype of *Aire*$^{-/-}$ mice was with *Card11*$^{unm/unm}$ (39). Normally, Card11 acts in the NFκB module of TCR-signaling, and this mutation leads to impaired Foxp3+ Treg differentiation in the thymus, 6–7 times fewer peripheral Tregs, and a gradual increase in Th2 cells (98). Interestingly, however, in *Aire*$^{-/-}$ mice, these low-frequency Tregs could still reduce tissue infiltration. Furthermore, while Tregs are crucial for controlling autoimmunity against several organs, they seem to play no prominent role in eye disease: *FoxP3*-mutant scurfy mice do not develop spontaneous uveitis, suggesting that other tolerance mechanisms are more important than Tregs in protecting against retinal autoimmunity.

Also very informative are the crosses of B6.*Foxp3*^{sf} mice (with the null "scurfy" *Foxp3* gene mutation) onto the *Aire*$^{-/-}$ mice or NOD genetic backgrounds (99). The *Sf* mutation by itself causes characteristic skin disease, massive lymphoproliferation, and infiltration most severely in the liver, but also the lungs and exocrine pancreas (100, 101). The crosses onto both backgrounds started to develop more severe lung and liver infiltrates much earlier and died significantly younger than B6.*Foxp3*^{sf} mice (99). While there were no changes in the infiltrates characteristically seen in other organs in B6.*Foxp3*^{sf} mice, those typical of *Aire*$^{-/-}$ mice on the C57BL/6 background (in the eyes, salivary glands) were – surprisingly – not seen in the B6.*Foxp3*^{sf} Aire-deficient mice. Moreover, phenotypes were identical in *sf* mutant mice on these *Aire*$^{-/-}$ and NOD backgrounds; to us, that implicates prolonged neonatal LIP rather than deficiency in thymic negative selection in this aggravated pathology in both crosses. *Sf* mutant Tregs are evidently not able to limit the activation of homeostatically proliferating T-cells. This is also illustrated by the similar wasting disease (with infiltrates in lungs, liver, pancreas, and stomach) in a model where neonatal T-cells are unable to respond to TGF-β signaling (102).

WHAT IS TRIGGERING AUTOIMMUNITY IN APECED PATIENTS?

If the mild phenotypes in *Aire*$^{-/-}$ mice are in line with the requirements for pathogenic T-cell activation, why are the phenotypes so much more severe in APECED patients? In humans too, it seems very unlikely that defective negative selection is the only cause of the severe autoimmune destruction of endocrine glands and other tissues (6, 21, 103). We are born with a much more mature immune system than mice (104, 105). Although lymphocyte function is under-developed in neonates, their numbers per milliliters of blood are even higher than in adult humans. Therefore, even if thymocyte migration is delayed because of impaired chemokine secretion by AIRE-deficient mTECs in the human fetus, this is probably compensated by the longer gestation. Neonatal lymphopenia has not been studied in APECED because the disease is usually diagnosed much later. Interestingly though, adult APECED patients have increased IL-7 concentrations in their sera that may be related to impaired T-cell homeostasis (106). The clear differences in disease phenotypes between APECED patients and *Aire*$^{-/-}$ mice suggest separate precipitating factor(s) in humans. These remain unidentified, but the surprisingly similar autoantibodies in patients with APECED and thymoma make any contribution from lymphopenia in human AIRE-deficiency seem even less likely (107). Nevertheless, the same logic – that additional activation is required before the rare naïve auto-reactive cells that escape from human AIRE-deficient thymi/thymomas can induce autoimmune disease – must apply in humans too (6, 103). In APECED, CMC, hypoparathyroidism, and Addison's disease sometimes present even at 2–3 years of age (4). Evidently, T-cells must go onto attack very soon after birth to destroy sufficient tissue to cause disease so soon; to us, that argues against any need for environmental triggers. Moreover, the first targets of the autoimmune attack are not AIRE-dependent TSAs (21). We propose that the pathogenic T-cells are already primed before their export from AIRE-deficient thymi or thymomas. A study on T-cells in APECED adults has shown gross alterations, especially in the CD8+ population, that include increased proliferation, lower expression of both IL-7R and the negative regulator of TCR-signaling CD5, and also absence of the regular naïve T-cell compartment, relative to

age-matched healthy controls (106). That could be secondary to the autoimmune diseases in APECED, a possibility that could be tested by assessing the activation of recent thymic emigrants before onset of APECED in pre-symptomatic young siblings of known patients.

In APECED, autoantibodies neutralizing type I IFNs and IL-22 can reach high titers even by 7 months of age, when autoantibodies to steroidogenic enzymes may also start to appear (108). Moreover, these autoantigens are produced in the thymus by cell types other than mTECs, so they should be available for negative selection even when AIRE is deficient (103). To explain these peculiarities, we have suggested biased selection or active autoimmunization in human thymi rendered "dangerous" by AIRE-deficiency (21, 103). That even leads to other secondary lymphoid tissue behavior in thymomas such as spontaneous production of anti-IFN-α and IL-12 autoantibodies by terminal plasma cells in sero-positive patients (109).

FURTHER PREDICTIONS

If gastritis in BALBc mice and EAU in B10.RIII mice are caused by LIP, they should be ameliorated by blocking homeostatic cytokines postnatally and simultaneously transferring lymphocytes into the lymphopenic hosts. As these cytokines sensitize TCRs through induction of pERK1/2, its inhibitors could be tested instead (65).

The phenotype of Cbl-b- and Aire double deficient mice could be mimicked by crossing with other mutant mouse strains with impaired T-cell susceptibility to anergy induction, or by thymectomizing *Cbl-b*$^{-/-}$ mice on days 1–3.

Curiously, autoimmunity is more often related to lower than higher TCR-signaling, perhaps because of weaker peripheral tolerance (65, 86). During their development, cortical thymocytes are positively selected when their receptors are triggered by self-peptide-MHC complexes. These so called "tonic" signals are also needed for T-cell survival in the periphery, but they are regulated to remain just below the threshold for activation and proliferation (62). When TCR-signaling is impaired, the cells have to adapt to respond to weaker signals, which makes them more responsive to self-antigens, e.g., during periods of over-production of homeostatic cytokines. Theoretically, crosses of *Aire*$^{-/-}$ mice onto backgrounds with decreased TCR-signaling and reduced T-cell survival could lead to phenotypes similar to those in *Aire*$^{-/-}$ × NOD crosses.

SUMMARY

It is unlikely that defective negative selection of auto-reactive thymocytes in AIRE-deficient thymi is the only cause of the associated autoimmune diseases in either model mice or APECED patients. Naïve T-cells require activation before they can cause tissue destruction: in uninfected neonates with no danger signals, tolerization by peripheral mechanisms seems a much likelier outcome. A hitherto under-recognized feature of *Aire*$^{-/-}$ mice is their prolonged neonatal lymphopenia: by inducing LIP, it favors the proliferation and activation particularly of auto-reactive T-cells. This also helps to explain the strikingly similar phenotypes of lymphopenic day 3 thymectomized and *Aire*$^{-/-}$ mice. However, the many developmental (ontogenetic) differences make LIP seem a much less likely contributor in humans – where we propose that additional mechanisms promote the early and much more sharply focused autoimmune attack on such unusual targets as the parathyroids, steroidogenic tissues/enzymes, and cytokines.

The mouse model has been extremely valuable in demonstrating Aire's role in negative selection of auto-reactive thymocytes. However, the differences in pathogenetic mechanisms and in autoimmune phenotypes in APECED patients question its suitability for testing new treatment options, and imply that merely restoring thymic TSA expression might not be enough to halt the autoimmunity in the patients. They also emphasize the importance of studies in human subjects, and again underline the need for caution when extrapolating from mouse models.

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Rag defects and thymic stroma: lessons from animal models

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Thymocytes and thymic epithelial cells (TECs) cross-talk is essential to support T cell development and preserve thymic architecture and maturation of TECs and Foxp3$^+$ natural regulatory T cells. Accordingly, disruption of thymic lymphostromal cross-talk may have major implications on the thymic mechanisms that govern T cell tolerance. Several genetic defects have been described in humans that affect early stages of T cell development [leading to severe combined immune deficiency (SCID)] or late stages in thymocyte maturation (resulting in combined immunodeficiency). Hypomorphic mutations in SCID-causing genes may allow for generation of a limited pool of T lymphocytes with a restricted repertoire. These conditions are often associated with infiltration of peripheral tissues by activated T cells and immune dysregulation, as best exemplified by Omenn syndrome (OS). In this review, we will discuss our recent findings on abnormalities of thymic microenvironment in OS with a special focus of defective maturation of TECs, altered distribution of thymic dendritic cells and impairment of deletional and non-deletional mechanisms of central tolerance. Here, taking advantage of mouse models of OS and atypical SCID, we will discuss how modifications in stromal compartment impact and shape lymphocyte differentiation, and vice versa how inefficient T cell signaling results in defective stromal maturation. These findings are instrumental to understand the extent to which novel therapeutic strategies should act on thymic stroma to achieve full immune reconstitution.

Keywords: thymus, Rag deficiency, Omenn and leaky SCID models, central tolerance, thymic reconstitution, thymic cross-talk

INTRODUCTION

Thymocytes and thymic epithelial cells (TECs) cross-talk is essential to support T cell development and preserve thymic architecture and maturation of TECs and Foxp3$^+$ natural regulatory T (nTreg) cells. In particular, deletion of self-reactive thymocytes in the thymic medulla is based on the recognition of self-antigens that are presented by medullary TECs (mTECs) and thymic dendritic cells (DCs). In this process, a key role is played by the autoimmune regulator (AIRE), a transcription factor expressed by a subset of mature mTEC that drives the expression of tissue-restricted antigens (TRAs), thus mediating negative selection of autoreactive thymocytes (1, 2). In addition, mature TECs from the Hassall corpuscles secrete thymic stromal lymphopoietin (TSLP), a cytokine that acts through thymic DCs and activates them to instruct self-reactive T cells to be diverted into Foxp3$^+$ nTreg cells (3). These findings highlight the critical role played by the thymus not only in the generation of a diversified and functional T cell repertoire, but also in the prevention of autoimmune manifestations. To this end, defects in T cell development represent a valuable model for studying mechanisms by which severe impairment in thymopoiesis may impinge on thymic stromal cell homeostasis and deletional and non-deletional mechanisms (4). In particular, severe combined immune deficiency (SCID) includes a heterogeneous group of genetic disorders that abolish T cell development at early stage of T cell differentiation by affecting survival of lymphoid progenitors (as in adenosine deaminase deficiency and reticular dysgenesis), interleukin (IL)-mediated expansion of lymphoid progenitors (as in patients with mutations in the γ common chain, JAK3, or IL-7 receptor), V(D)J recombination [as in recombination activating gene (Rag)1 and 2, and Artemis deficiency] in lymphoid precursors and signaling through the pre-T cell receptor (mutations of CD3δ, CD3ε, CD3ζ, and CD45) (5). Null mutations in these SCID-causing genes are associated with a virtual lack of circulating T lymphocytes. However, hypomorphic mutations in the same genes may allow for development of a restricted number of T lymphocytes with limited repertoire diversity, which, when exported to the periphery, may infiltrate target tissues and cause autoimmunity and organ dysfunction, as in patients with Omenn syndrome (OS) (6–10). Finally, defects in T cell development that compromise thymocyte development beyond the CD4$^+$ CD8$^+$ [double-positive (DP)] stage result in combined immunodeficiency with residual number of circulating T lymphocytes that show abnormal phenotype and function. In particular, impaired production of single-positive (SP) CD4$^+$ cells is observed in major histocompatibility complex (MHC) class II deficiency, whereas generation of SP CD8$^+$ lymphocytes is compromised in ZAP70

deficiency (11, 12). Association of these conditions with immune dysregulation has been reported, although not as frequently as in OS due to hypomorphic mutations in SCID-causing genes (13).

In this review, we will focus the discussion on the contribution of thymic microenvironment on the pathogenesis of peripheral immune pathology in the presence of residual V(D)J recombination activity. To this end, we will discuss findings observed in $Rag2^{R229Q/R229Q}$ and $Rag1^{S723C/S723C}$ mutant mice, which represent a valuable model of OS and atypical SCID, respectively (14–18). Collectively, we provide evidence that abnormalities of thymic stroma secondary to impaired development of T lymphocytes may affect key mechanisms of immune tolerance and ultimately result in severe manifestations of immune dysregulation.

MOUSE MODELS OF LEAKY SCID AND OS

Mutations of Rag genes result in a variety of clinical and immunological phenotypes. In particular, while null mutations cause a severe block in T and B cell development (T^- B^- SCID), hypomorphic *Rag1* and *Rag2* mutations may cause a spectrum of phenotypes, including OS, atypical SCID, combined immune deficiency with expansion of TCRαβ$^+$ T cells, and combined immune deficiency with granuloma and/or autoimmunity (CID-G/A) despite their common molecular mechanisms underlying the disease (19–25). While all of these conditions associated with hypomorphic *Rag* mutations are characterized by residual development of T (and in some cases, B) lymphocytes, some of them (especially OS and CID-G/A) present with prominent immune dysregulation. However, the cellular and molecular mechanisms underlying autoimmunity have remained poorly defined until recently, when animal models of OS and leaky SCID have become available (16, 17, 26). In particular, Khiong and colleagues have reported on a spontaneously occurring mouse mutant (named MM) in which a homozygous point mutation in the *Rag1* gene (R972Q) was associated with a high proportion of memory T cells in the periphery. Although MM mice showed skin redness when shaved, no T cells infiltration was observed in the tissues and no obvious signs were reported, making this mutant strain a model of leaky SCID, in which T and B cells are present in low number and T cells are predominantly activated, but no obvious signs of autoimmunity are present (26). In another mouse model, homozygosity for the Rag1 S721C mutation was associated with impaired T cell development, presence of oligoclonal, activated T cells, profound B cell lymphopenia, and yet significant serum levels of immunoglobulin (15, 17, 18). Although only a minority of $Rag1^{S723C/S723C}$ mice developed signs of OS, T cell infiltrates in peripheral tissues, and autoantibodies to double stranded DNA (dsDNA) and other self-antigens were demonstrated in a significant proportion of mutant mice 15. Immune dysregulation was even more prominent in another mutant mouse model carrying a homozygous *Rag2* R229Q mutation, as shown by expansion of oligoclonal activated T cells infiltrating target organs including skin, gut, liver, and lung and by the presence of high IgE serum levels and autoantibodies, despite the absence of circulating B cells (14, 16). Of note, immune dysregulation in $Rag1^{S723C/S723C}$ and $Rag2^{R229Q/R229Q}$ mutants was associated with profound thymic abnormalities, with lack of corticomedullary demarcation (CMD), and impaired maturation of TECs (17, 27).

In particular, both $Rag1^{S723C/S723C}$ and $Rag2^{R229Q/R229Q}$ mice displayed altered maturation of mTECs, as indicated by the virtual absence of expression of claudin-4 (Cld4) and Ulex europaeus Agglutinin 1 (UEA-1) ligand. Furthermore, analysis of cytokeratin (CK) expression in the thymus revealed abundance of CK8$^+$ CK5$^+$ cells, which represent immature TEC progenitors and a severe reduction of CK8$^-$ CK5$^+$ mTECs. FACS analysis labeling CD45$^-$ Epcam$^+$ thymic stromal cells with UEA-1 and Ly51 specific antibodies for mTECs and cTECs, respectively, have demonstrated the increased frequency of cTECs with consequent reduction in mTEC compartment in $Rag2^{R229Q/R229Q}$ mouse compared to WT (**Figure 1A**). However, all epithelial populations were significantly diminished in number given the dramatic reduction in total thymic cellularity (**Figure 1B**). Defective maturation of mTECs in $Rag1^{S723C/S723C}$ and $Rag2^{R229Q/R229Q}$ mice was associated with severe reduction of AIRE-expressing cells and markedly reduced expression of TRAs, such as cytochrome P450, insulin 2, glutamic acid decarboxylase 67, and fatty acid-binding proteins (17, 27). These defects inevitably lead to a severe impairment in the process of negative selection of autoreactive T cells clones.

Unexpectedly, abnormalities of thymic DCs, which are involved in promoting negative selection of self-reactive thymocytes and in the generation of nTregs, were also demonstrated in both mutant models. In particular, a relative abundance of CD11c^{int} CD45RA$^+$ plasmacytoid DCs (pDCs), and a decreased proportion of CD11c$^+$ CD45RA$^-$ conventional DCs (cDCs) was demonstrated in $Rag1^{S723C/S723C}$ mice (17). A severe reduction of both cDCs and pDCs was demonstrated in $Rag2^{R229Q/R229Q}$ mice, and was associated with a random distribution of these DC subsets throughout the thymus. Furthermore, a significant reduction in the expression of MHC-II and CD86 was found in both DC subset populations, suggesting impairment in DC maturation process (28). Of note, impaired maturation of mTECs, defective expression of AIRE and reduced number of thymic DCs have been also reported in patients with hypomorphic mutations of genes involved in early stages of T cell development (29, 30). While the mechanisms accounting for thymic DC abnormalities in mice and patients with hypomorphic *Rag* mutations remain poorly defined, they have important consequences on maintenance of immune homeostasis. In particular, cDCs have been described to contribute to the generation of nTregs (31). Consistent with this, a reduced number of Foxp3$^+$ nTreg cells have been observed in both $Rag1^{S723C/S723C}$ and $Rag2^{R229Q/R229Q}$ mice, as well as in patients with Rag-dependent OS (16, 17, 30).

Altogether, the study of animal models carrying hypomorphic *Rag* mutations has demonstrated that defective T cell lymphopoiesis affects maturation and function of thymic stroma, and impinges on both deletional and non-deletional mechanisms of immune tolerance, thereby providing important insights on the pathophysiology of OS.

ANIMAL STUDIES TO TARGET THYMIC STROMA IN Rag DEFICIENCIES

GENE THERAPY IN *Rag1* KNOCK-OUT MICE

An additional demonstration of the importance of thymic lymphostromal cross-talk has been provided by recent data

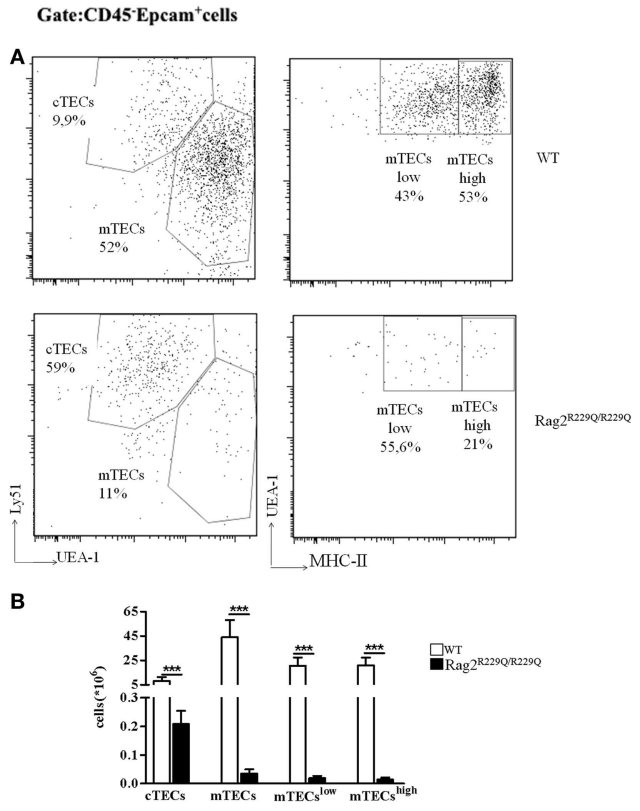

FIGURE 1 | Thymic epithelial cell alterations in *Rag2*$^{R229Q/R229Q}$ mouse. **(A)** Representative FACS analysis of thymic epithelial compartment defined as Epcam$^+$ cells in the CD45 negative fraction obtained after enzymatic digestion and enriched through AUTOMACS selection. Digested cells were stained with specific antibodies (Ly51, UEA-1, and MCH-II) to identify the different epithelial subsets as indicated in the dot-plots. Numbers represent the percentage within the indicated regions. **(B)** Graphic representation of absolute numbers for each epithelial population obtained upon enzymatic digestion in all mice analyzed (WT, $n = 5$; *Rag2*$^{R229Q/R229Q}$, $n = 9$). Groups were analyzed with Prism software (GraphPad) using a two-tailed Mann–Whitney unpaired test. Data are presented as mean ± SD. *P*-values of <0.05 were considered significant.

demonstrating that inefficient T cell reconstitution following gene therapy in *Rag*1 deficient mice results in an OS-like phenotype (32). In this particular model, the majority of *Rag1* knock-out (KO) mice treated with lentiviral vectors carrying codon optimized human Rag1 cDNA driven by ubiquitous and cell type-restricted promoters showed low level of T cell reconstitution. In this setting of T cell lymphopenia, homeostatic proliferation led to peripheral T cell expansion, associated with a restricted T cell repertoire and a tendency of T cells to infiltrate peripheral tissues such as skin, lung, and kidney. Impaired T cell reconstitution, with reduced thymic cellularity, led to only partial rescue of thymic stroma morphology and maturation, with focal areas of CMD and low number of mature mTECs expressing AIRE. By contrast, transplantation of wild-type bone marrow cells into *Rag1*$^{-/-}$ mice leads to the rescue of thymopoiesis and thymic stroma architecture, with presence of a well-defined CMD and a normal distribution of cTEC, immature and mature TEC expressing UEA-1 ligand and AIRE. Of note, 2 months after treatment, 50% of gene therapy-treated mice started to develop skin rash and wasting syndrome, which in some cases led to death. Poor thymic reconstitution correlated with massive lymphocytic infiltrates in peripheral tissues and presence of activated (CD44$^+$CD69$^+$) T cells, despite the presence of Foxp3$^+$ cells. Moreover, gene therapy-treated mice showed a significant increase in serum IgE levels, presence of anti-dsDNA antibodies and increased BAFF levels, which represent typical biomarkers of immune dysregulation in patients and animal models of OS (14, 18). These data indicate that inadequate rescue of Rag1 expression leads to poor reconstitution of T and B cells and is insufficient to restore thymic stroma architecture, maturation of AIRE and TSA-expressing mTECs, and induction of both T and B cell tolerance. In this scenario, development of a limited number of T and B lymphocytes and inability to maintain efficient tolerance checkpoints lead to the development of OS-like manifestations. Overall, these data illustrate the importance of T cell reconstitution for restoring the differentiation and maturation of TECs, and emphasize the relevance of thymic stroma in ensuring immune tolerance and preventing thymic egress of autoreactive T cell clones.

ANTI-CD3ε mAb TREATMENT IN Rag2$^{R229Q/R229Q}$ MOUSE MODEL OF OS

As previously described, OS is an atypical SCID in which the coexistence of immunodeficiency and autoimmunity remains an intriguing aspect that needs to be further investigated. Thanks to availability of the *Rag2*$^{R229Q/R229Q}$ mouse model, we have studied various mechanisms that contribute to the pathogenesis of autoimmune manifestations of OS. We have demonstrated that in addition to hypomorphic *Rag* defect leading to generation of a limited number of T cells, severe defects in thymic epithelial compartment occur, which contribute to the escape of autoreactive T cells that invade the periphery triggering autoimmunity (24). This model represents also a valuable tool to evaluate the effects of TCR signaling on maturation of the thymic stromal compartment. To this end, we evaluated the *in vivo* effect of anti-CD3ε monoclonal antibody (mAb) administration in neonatal and adult mice. While no significant changes were noticed in the thymus of adult treated mice, injection of anti-CD3ε mAb at neonatal age resulted in a dramatic amelioration of the epithelial compartment and peripheral immunopathology. In particular, treatment was associated with a marked reduction in the frequency of effector/memory T cells in the periphery and a significant decrease in interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production by peripheral T cells. These changes were paralleled by significant modification in thymus morphology, with appearance of distinct areas of CMD and significant improvement of the medullary/cortical ratio (27). Double staining for CK5 and CK8 further confirmed these findings by revealing the presence of well-defined cortical and medullary areas showing that anti-CD3ε mAb treatment enforces maturation of TECs leading to compartmentalization of CK8$^+$CK5$^-$ cTECs and CK8$^-$CK5$^+$ mTECs (**Figure 2A**). Moreover, we have described an increase in the presence of UEA-1$^+$ cells, although the formation of UEA-1$^+$ mature mTECs clusters was not fully restored.

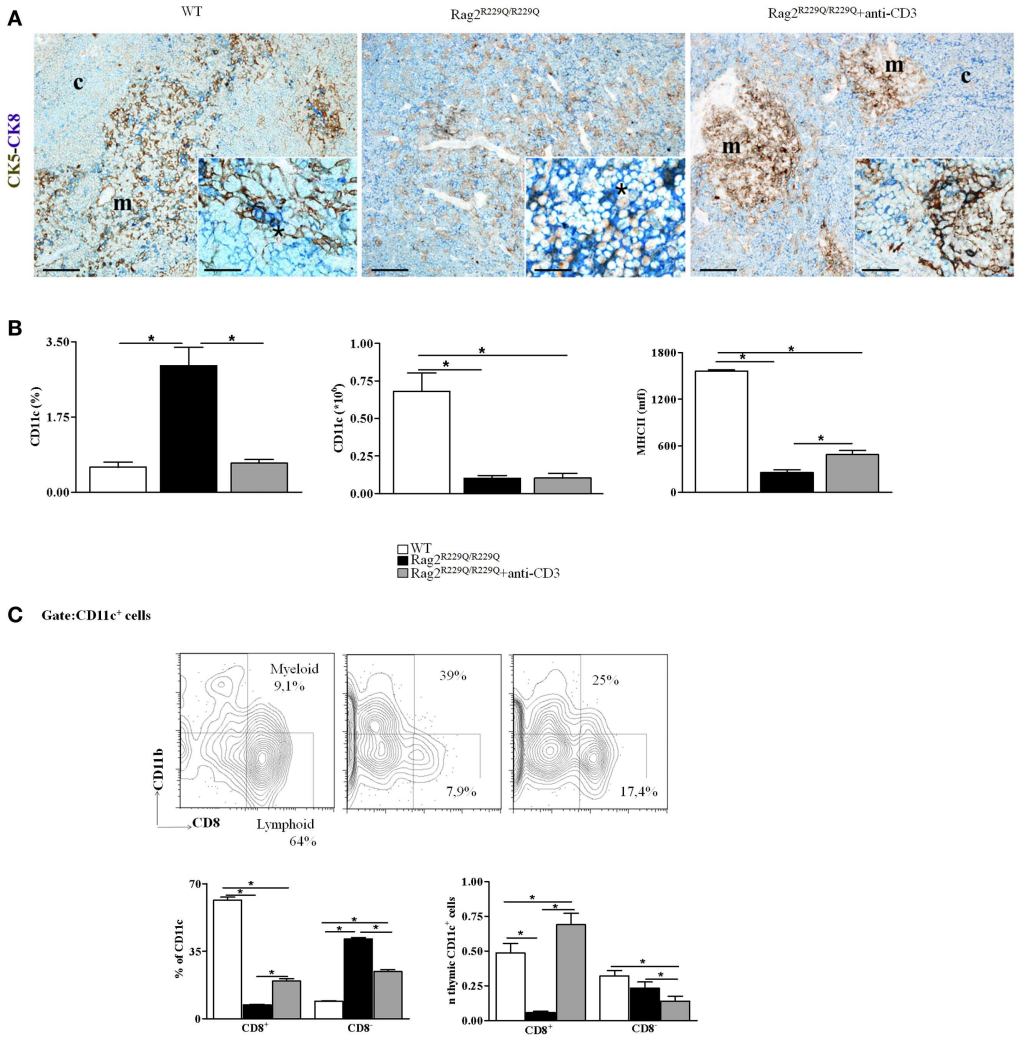

FIGURE 2 | Anti-CD3ε mAb administration enhances thymic epithelium compartmentalization and maturation and modifies thymic DCs frequency and distribution in *Rag2*$^{R229Q/R229Q}$ newborns. (A) Left panel shows representative immunohistochemistry from WT thymus displaying a well-defined corticomedullary differentiation and normal compartmentalization of CK8$^+$CK5$^-$ cTECs (CK8, blue) and CK8$^-$CK5$^+$ mTECs (CK5, brown). mTECs show mature morphology with large cytoplasm and delicate CK5 positivity with rare double-positive CK8$^+$CK5$^+$ immature TECs disposed along the corticomedullary junction (asterisk within inset). Corticomedullary differentiation and maturation of TECs are profoundly impaired in *Rag2*$^{R229Q/R229Q}$ mouse (middle panel) in which immature TECs expressing both CK5 and CK8 are highly represented (asterisk within inset). Anti-CD3ε mAb administration enforces maturation of TECs leading to compartmentalization of CK8$^+$CK5$^-$ cTECs and CK8$^-$CK5$^+$ mTECs (right panel), although mTECs are still closely packed and irregularly distributed with intense CK5 positivity as compared to the normal medulla (detail of morphology within inset). Double immunohistochemical staining: CK5 (brown staining) and CK8 (blue staining). (m, medulla; c, cortex; scale bars corresponds to 200 and 50 μm for 10× and 40× (insets) original magnification, respectively). **(B)** Graphic representation of the percentage and absolute number of CD11c$^+$ cells in the thymus of all mice analyzed (WT, $n = 7$; *Rag2*$^{R229Q/R229Q}$, $n = 7$; *Rag2*$^{R229Q/R229Q}$ + anti-CD3 $n = 9$). The last graph on the right indicates mean fluorescence intensity (MFI) of MHC-II expression on total CD11c$^+$ cells in all mice analyzed (WT, $n = 5$; *Rag2*$^{R229Q/R229Q}$, $n = 6$; *Rag2*$^{R229Q/R229Q}$ + anti-CD3 $n = 4$). **(C)** Representative dot plot indicating the distribution of myeloid (CD8$^-$) and lymphoid (CD8$^+$) populations in the gate of CD11c$^+$ cells (upper panel). Statistics of the percentage and the absolute numbers of CD8$^+$ and CD8$^-$ CD11c$^+$ in all mice analyzed (WT, $n = 5$; *Rag2*$^{R229Q/R229Q}$, $n = 4$; *Rag2*$^{R229Q/R229Q}$ + anti-CD3 $n = 4$) (lower panel). Groups were analyzed with Prism software (GraphPad) using a two-tailed Mann–Whitney unpaired test. Data are presented as mean ± SD. *P*-values of <0.05 were considered significant.

Furthermore, treatment with anti-CD3ε mAb normalized the frequency while did not change the absolute number of total thymic DCs and significantly increased MHC-II expression in this population normally down-regulated in *Rag2*$^{R229Q/R229Q}$ mice respect to WT counterpart (**Figure 2B**). More interestingly, anti-CD3ε mAb treatment induced a redistribution of the two thymic DCs main subsets CD8$^-$ (myeloid) and CD8$^+$ (lymphoid) (**Figure 2C**). The improvement of thymic stroma architecture and maturation were associated with a reduction in tissue infiltrates, as demonstrated by the reduced frequency of CD4$^+$ and CD8$^+$ cells in the skin, gut, lung, and liver. Altogether, these data indicate that treatment with anti-CD3ε mAb has a beneficial effect

on thymic stroma and on peripheral immunopathology, and may pave the way for similar therapeutic modalities aiming at improving immune function and reducing signs of immune dysregulation in patients with SCID characterized by poor thymic maturation, while waiting for definitive treatment based on hematopoietic cell transplantation.

CONCLUSION

Thymocytes and TEC cross-talk are fundamental for the maintenance of thymic architecture and function. Investigation on thymic morphology and immunophenotype in SCID patients and in parallel analysis of murine models of OS and Leaky SCID have revealed the extent to which altered thymic cross-talk might lead to immune dysregulation and ultimately cause peripheral immunopathology. Thymic stromal improvement and amelioration of peripheral immunopathology upon anti-CD3ε mAb administration in OS mouse model have further highlighted the contribution of thymic stroma in the pathogenesis of immune dysregulation. In parallel, poor immunological reconstitution observed in the preclinical study of gene therapy caused by inadequate Rag1 expression has further emphasized the relevance of stromal thymic compartment in the induction and maintenance of immune tolerance. Overall these findings further define the role of thymic epithelium in immune reconstitution and indicate that cTECs and mTECs full restoration has to be achieved to prevent immune dysregulation.

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Corrigendum: Rag defects and thymic stroma: lessons from animal models

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