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# Human thymus in health and disease: recent advances in diagnosis and biology

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## Abstract

The thymus is the crucial tissue where thymocytes develop from hematopoietic precursors that originate from the bone marrow and differentiate to generate a repertoire of mature T cells able to respond to foreign antigens while remaining tolerant to self-antigens. Until recently, most of the knowledge on thymus biology and its cellular and molecular complexity have been obtained through studies in animal models, because of the difficulty to gain access to thymic tissue in humans and the lack of *in vitro* models able to faithfully recapitulate the thymic microenvironment.

This review focuses on recent advances in the understanding of human thymus biology in health and disease obtained through the use of innovative experimental techniques (eg. single cell RNA sequencing, scRNAseq), diagnostic tools (eg. next generation sequencing), and *in vitro* models of T-cell differentiation (artificial thymic organoids) and thymus development (eg. thymic epithelial cell differentiation from embryonic stem cells or induced pluripotent stem cells).

### Keywords

Thymus; T-cell development; Inborn errors of immunity; T-cell lymphopenia; Thymic development; Thymic stroma

## 1. Introduction

The thymus is the lymphoid organ specialized in T cell development; it contains cells of different embryonic origin that form a meshwork with a well-defined tissue architecture [1, 2]. During embryogenesis, the thymus primordium originates together with the parathyroid

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glands from the third endodermal pharyngeal pouches (PP), surrounded by neural crest cells (NCC) [3]. The main genetic regulatory network driving embryonic thymus development comprises a series of transcription factors including HOXA3, PAX1, PAX9, EYA1, SIX1 and TBX1 [4, 5]. These genes are regulated by signaling molecules released by the NCC, including retinoic acid (RA), proteins of the Wingless-int (WNT) family, bone morphogenic proteins (BMP), fibroblast growth factors (FGF) and sonic hedgehog (SHH) proteins (Reviewed in [6]). All these molecules are involved in driving the development of the thymic primordium, starting from the third PP, while HOXA3 and EYA1, and possibly CHD7, play also a role in the development of neural crest derived mesenchymal cells [7]. A recent report in mice has indicated the transcription factor Foxi3 as a new player in the pathway involved in thymus organogenesis [8]. FOXI3 is expressed in the third PP endoderm and mice FOXI3-null lack thymus and parathyroid glands. FOXI3 appears to act downstream of TBX1 and regulates PAX9, but it might interact also with HOXA3 [8]. All these first steps of thymus organogenesis are independent from *FOXN1* expression, while the second phase of thymus development is FOXN1-dependent [9]. In this phase, FOXN1 induces the expression of several target genes that play a crucial role in thymocyte recruitment (eg. DLL4, CXCL12, CCL25) and thymic epithelial cell (TEC) maintenance and differentiation in cortical TEC (cTEC) and medullary TEC (mTEC) [10–13].

The main cellular component of the thymus are the developing thymocytes, which derive from bone marrow hematopoietic stem cells that continuously colonize the thymus, entering through blood vessels at the cortico-medullary junction [4, 5]. Other cell types of hematopoietic origin also reside in thymus and include dendritic cells (DC), natural killer cells (NK) and B cells, but these represent a minimal fraction of cells when compared to developing T cells. Non-hematopoietic stromal cells represent another important cell component of the thymus and are mainly represented by TECs, which are the cell type studied more in detail over the years. TECs can be divided in two main subsets, cortical (cTECs) and medullary TECs (mTECs), which share the same endodermal origin but play different roles and are located in distinct areas within the thymus [14]. In particular, cTECs are involved in the early phases of thymocyte development, which take place in the thymus cortex, and actively participate in the positive selection leading to the generation of $CD4^+$ $CD8^+$ double positive (DP) cells. The thymocytes then move to the medulla, where they complete their maturation to $CD4^+$ or $CD8^+$ single positive (SP) cells and undergo negative selection to eliminate self-reactive T cells. The mTECs play a crucial role in supporting the later stages of thymocyte development and in particular a specialized subset of mTECs mediate the negative selection process, through the expression of tissue restricted antigens (TRAs) induced by the transcriptional activator AutoImmune Regulator (AIRE). TRAs are presented to SP thymocytes; among these, SP cells with high affinity to self-antigens are eliminated or differentiate into regulatory T cells (Tregs), while thymocytes with low affinity to self-antigen survive and leave the thymus with a phenotype of naïve or recent thymic emigrant (RTE) cells to reach the peripheral tissues.

## 2. Recent advances in human thymus biology

The study of the human thymus has been hampered for many years not only because of the limited availability of this tissue, but also because of the lack of experimental techniques

able to dissect the complexity of the thymus. Indeed, we know that the thymus is composed of many cell types; studies using flow cytometry, histology or bulk RNA sequencing (RNAseq) could only capture in part this complexity, leaving many questions unanswered. The more extensive knowledge on human hematopoietic cell markers previously allowed a more detailed dissection of these cells in human thymus, when compared to the stromal cell compartment. However, in the last few years, use of innovative techniques such as single cell RNA sequencing (scRNAseq) and spatial transcriptomics have provided a tremendous amount of new insights into the complexity of this tissue, both in hematopoietic and stromal cell compartments. Indeed, several studies [15–18] have indicated that the diversity and complexity of cTEC and mTEC subsets is much greater, although the function and spatial localization of the novel described TEC subsets has yet to be fully understood. Recently, there has also been a better appreciation of the importance of other non-epithelial stromal cell types, including endothelial cells, fibroblasts and other mesenchymal cells, in the development and function of the thymus [2, 4, 17, 19, 20]. All these cell types contribute to correct function of the thymus and their interactions, also called “lympho-stromal cross-talk” are crucial for the correct development and function of the thymus and for the generation of a diverse and self-tolerant repertoire of T lymphocytes.

Park and colleagues [16] generated a human thymus cell atlas using dissociated cells from 15 prenatal samples (ranging from 7 to 17 post conception weeks) and 9 post-natal tissues (ranging from 3 months to 35 years). Most of the cells in these samples were sorted using the hematopoietic cell markers CD45 or CD3, while only 3 samples were enriched using the epithelial cell marker EpCAM, prior to the single cell capture. In their dataset, the authors identified more than 40 different cell types or cell states, which expressed specific marker genes. In particular, they showed that cell states in the thymus dynamically change in terms of abundance and gene expression profiles through the development from fetal to post-natal life. The authors were able to establish computationally the trajectory of human T cell development from early progenitors to the different subsets of mature T cell types; using this trajectory, they built a list of putative transcription factors that guide T cell determination. Moreover, they identified a novel subset of unconventional T cells, among the CD8αα T cell subset, characterized by the expression of GNG4 and located in the peri-medullary region of the thymus. They also defined novel subpopulations of thymic fibroblasts and epithelial cells, and characterized their tissue location. Two different subsets of thymic fibroblasts (Fb) were identified in this study, expressing distinct gene sets and likely playing different roles: Fb1 cells express genes such as *COLEC11* and *ALDH1A2* and are postulated to play a role in sustaining epithelial cell growth, while Fb2 cells express extracellular matrix genes and semaphorins and are supposed to participate in vascular development. Finally, the authors identified several subsets of TECs. A previous study using scRNAseq in murine TECs by Bornstein and colleagues [15] had shown for the first time that the complexity of TECs had been greatly underestimated until then. This study also described the presence of 4 distinct sub-populations of mTECs. Using the annotations from Bornstein and colleagues’ murine dataset, Park and colleagues were able to discover the presence of conserved subsets of TECs across species, including *PSMB11*-positive cortical TEC (cTEC), *KRT14*-positive mTECI, *AIRE*-positive mTECII and *KRT1*-expressing mTECIII. They also distinguished a subset of mTEC expressing the markers *POU2F3* and *DCLK1*,

listed as the specific markers of the Tuft-like mTECIV subset newly identified in murine thymus [15, 21], although in the human thymus these markers are not uniquely expressed by Tuft-like mTECs. Finally, this study identified for the first time in humans clusters of TECs that were identified as neuroendocrine and myoid cells, respectively, based on their gene expression profile. This report provided for the first time evidence of the complexity of the stromal compartment in the human thymus; however, stromal cells represented only the minority of the single cell dataset, since the $EpCAM^+$ cell enrichment was performed in few samples [16]. A subsequent study by Bautista and colleagues [17] aimed at dissecting in greater detail the cellular heterogeneity of the human stromal cell compartment. In this manuscript, the authors performed scRNAseq on enriched CD45-negative cells isolated after enzymatic digestion from 5 human thymic samples ranging from fetal to adult. They identified several stromal clusters, including epithelial, mesenchymal, pericytic, endothelial and mesothelial cell clusters. Gene expression analysis revealed that mesenchymal cell clusters expressed many ligands and regulators of WNT, BMP, Transforming Growth Factor beta (TGF-β), Insulin-like Growth Factor (IGF), and Fibroblast Growth Factor (FGF) signaling pathways, which have been all shown in mouse models as crucial in sustaining development and function of TECs, which express specific receptors that bind such factors [22–29]. Additionally, expression of some of the ligands, such as KGF, BMP4 and FRZB, in mesenchymal cells was increased in post-natal and adult samples, indicating a different role for these cells in supporting TEC development over-time. Pericytes and mesothelial cells were also found to express many ligands of the above-mentioned signaling pathways, while they were exclusively expressing genes such as *INHBA* (encoding for the subunits of Activin A) and the Activin antagonist follistatin (*FST*), respectively, both of which have been recently shown to play important roles in TEC differentiation and maintenance [23]. In summary, these data clearly show how these various non-epithelial stromal subsets play a complementary role in supporting human TEC development and function. On the other hand, endothelial cells were found to express extracellular matrix and adhesion molecules, such us fibronectin (FN1) and LGALS3, which could play a role in attracting and regulating migration of hematopoietic progenitors. In this report the authors were also able to explore in detail the complexity of the human TEC compartment and defined novel markers for some of the TEC subsets. Upon re-clustering of epithelial cells, they described two different clusters containing cells expressing cortical TEC (cTEC) markers, cTEChi and cTEClo, characterized by high and low levels of cTEC functional markers, respectively, and several other clusters expressing mTEC markers. In addition to the mTEC subsets already described by Park et al [16], such as $CCL21^+$ mTECs, $AIRE^+$ mTECs, $KRT1^+$ mTECs, neuroendocrine, and myoid mTECs, the authors identified novel clusters of TECs, such as myelin-expressing TECs, ciliated TECs and ionocytes. $CFTR^+$ ionocytes are particularly intriguing, since these cells had not been previously identified in the human thymus, whereas they are a well-known component of the lung epithelium, where they arise from basal cells and give rise to neuroendocrine and tuft-like cells. In the human thymus, all these cell subsets were shown to be located in close proximity in association with Hassall's corpuscles; this could suggest the presence in the thymus of a common progenitors for these subsets. The authors also introduced a novel subset of TECs, the immature TECs, which express TEC identity genes but lack genes characteristic of cTECs and mTECs, and may represent TECs that have lost their differentiated phenotype. In fact, immature TECs were

found especially enriched in the adult thymic sample, to the detriment of functional TECs. Characterization of this subset could be important to determine the genes and pathways playing a role in thymic involution. Another interesting hypothesis suggested by Bautista and colleagues is that in addition to AIRE$^+$ mTECs, other TECs likely participate in tolerance induction by expressing tissue restricted antigens (TRA), which could then be presented by antigen presenting cells, such as DC. They propose that myoid cells could play a role in inducing tolerance to muscle antigens, since they found that genes encoding for the acetylcholine receptor (CHRNA1) and titin (TTN), which are associated to the neuromuscular autoimmune disease myasthenia gravis are more abundantly expressed by myoid, ciliated and neuroendocrine cells as compared to AIRE-mTEC. Figure 1 shows the new subsets of TECs described in this section and lists some of their representative markers.

Of note, a report published by Campinoti and colleagues [30] has taken on the task of identifying and characterizing the still elusive epithelial stem/progenitor cells in human post-natal thymus. By culturing TEC obtained after enzymatic dissociation of human thymus, they were able to identify a subset of cells possessing the ability to expand extensively upon weekly passages. These cells could derive both from cortical and medullary TEC, were found to express high levels of CD49f and CD90, and presented a hybrid epithelial-mesenchymal phenotype. Most importantly, when these cells were combined with thymic interstitial cells in a rat thymic decellularized extracellular matrix structure, they were able to reconstitute a 3D structure reproducing the native thymus. The resulting scaffolds were also capable of supporting human T cell development from hematopoietic progenitors, both *in vitro* and *in vivo*. These hybrid epithelial-mesenchymal cells, acting as progenitor human post-natal stromal cells, could prove very useful for prospective applications aiming at thymus regeneration for the treatment of thymic defects.

Other interesting and novel observations on the human thymus where recently published in a human atlas integrating scRNAseq and spatial transcriptomic data from 9 immune organs collected pre-natally between 4 and 17 weeks post-conception [31]. This report confirmed that T cell progenitor cells are only found in fetal thymus, corroborating the notion that the thymus is absolutely necessary for the development of T cells and that, in conditions in which it is absent, there is complete T cell lymphopenia [32]. Another intriguing part of this study explored the origin of unconventional T cell subsets, which has not yet been fully clarified. Unconventional T cells were found to express the innate marker ZBTB16 (PLZF) and could be separated in 3 subsets: Type 3 innate T cells or Th17-like T cells (RORC$^+$ and CCR6$^+$), Type1 innate T cells or NKT cells (EOMES$^+$ and TBX21$^+$) and CD8$\alpha\alpha$ T cells. Interestingly, conventional mature T cells spatially co-localized with mTECs in the inner medulla, while CD8$\alpha\alpha$ T cells and Type 1 innate T cells co-localized with DC at the cortico-medullary junction. Regulatory T cells (Treg) and Type 3 innate T cells were found in both locations. CD8$\alpha\alpha$ T cells and Type 1 innate T cells may thus undergo negative selection processes mediated by DC and not by mTECs, as is the case for conventional T cells. Additionally, the authors proposed that also the process of positive selection could be different for unconventional T cells. Indeed, by evaluating the TCR repertoire in single T cells, they observed that usage of *V-J* genes within the T cell receptor $\alpha$ (TRA) locus in unconventional T cells had a pattern that was intermediate between what detected in double positive (DP) and conventional T cells, suggesting that fewer recombination rounds occur

in unconventional T cells before positive selection. These observations led the authors to hypothesize that unconventional T cells may originate after positive selection on neighboring DP T cells, as opposed to positive selection on cTECs as is the case for conventional T cells.

Importantly, all this extraordinary amount of novel knowledge on the complexity of the human thymus has been derived from studies performed on normal thymic samples. More limited data are available on thymic cell composition and spatial distribution in patients with defects of thymus development and function. This is because such defects are quite rare and, in most severe cases, no thymic tissue can be visualized and recovered. Moreover, ethical reasons limit availability of thymic tissues from patients in which the thymus is present, although reduced in size or not fully functional. Finally, post-mortem samples are often collected some time after death, and may not be adequate to perform studies such as scRNAseq. For these reasons, the only thymic tissues available from patients with thymic defects are limited to those in which the immunodeficiency is associated to a cardiac abnormality requiring cardio-thoracic surgery, eg. DiGeorge Syndrome (DGS), CHARGE Syndrome and Trisomy 21. In these cases, the thymus is removed in its entirety or partially at the time of surgery to gain access to the heart. While several studies have reported on abnormalities of thymus architecture in patients with these forms of immunodeficiencies [33–38], no reports of scRNAseq and/or spatial transcriptomics on thymic tissue obtained from patients carrying thymic defects have been published yet. Data obtained from these pathological samples will be extremely informative and crucial in providing additional insights into human thymus development and function.

## 3. Novel diagnostic tools to identify thymic defects

Limited availability of human thymic tissue from healthy individuals and from patients carrying genetic defects affecting thymus development and/or function represents a significant challenge to identify and characterize novel thymic defects. For these reasons, discovering novel genetic causes of thymic abnormalities has been extremely problematic, and many thymic defects remain undiagnosed even today. However, in the most recent years the increasing worldwide availability of newborn screening (NBS) for severe combined immunodeficiencies (SCID) using tests evaluating T Cell Receptor Excision Circles (TRECs) [39], has allowed the identification, very early in life, of patients with severe T-cell lymphopenia. Of note, in published reports, DGS and other thymic defects have been identified in about 1:20000–60000 infants with a positive TREC-based newborns screening assay; of these, complete athymia has been reported in about 5% of the cases [40–49]. However, the TREC assay does not identify all cases of 22q11.2del syndrome [50–52]. Next generation sequencing techniques, targeted to genes known to cause Inborn Errors of Immunity (IEI) or spanning across the whole exome (WES) or even the whole genome (WGS), have become more accessible to clinicians and affordable, allowing the discovery of a growing number of novel causes of thymus abnormalities (see Table 1). Several of these disorders are due to heterozygous gene and chromosomal defects, including 22q11.2del syndrome, *TBX1* and *FOXI3* deficiencies. In some cases, multiple inheritance patterns have been identified. For example, FOXN1 deficiency may occur as a fully penetrant autosomal recessive trait, or as a heterozygous condition with variable clinical penetrance. Importantly, thymus-intrinsic defects are often associated with multi-organ clinical manifestations.

Finally, non-genetic causes can lead to abnormalities of thymic development, most notably poorly controlled maternal diabetes [53, 54].

Recently, this approach allowed the identification of a novel cause of severe T-cell lymphopenia at birth caused by a defect in thymic stromal cells: *FOXN1* haploinsufficiency [55, 56]. In particular, we and others have described a series of pediatric patients with marked T cell lymphopenia and low TREC levels at birth, who were found to carry heterozygous loss-of-function *FOXN1* variants. *FOXN1* is a master gene regulator of TEC function; bi-allelic loss-of-function variants in the *FOXN1* gene lead to the nude/SCID phenotype, characterized by thymic aplasia, alopecia and nail dystrophy [57]. This is a severe condition that requires thymus implantation. By contrast, clinical and immunological abnormalities tend to improve spontaneously over-time in individuals with heterozygous loss-of-function *FOXN1* variants, so that definitive therapeutic interventions are not required in the majority of these subjects. Three of the subjects included in our report underwent hematopoietic stem cell transplantation (HSCT) before being diagnosed with *FOXN1* haploinsufficiency, but none of them had clinical benefit and one died from complications related to the transplant [55], consistent with the notion that *FOXN1* haploinsufficiency causes a thymus stromal intrinsic defect that cannot be corrected by HSCT.

Using the same approach, a few years ago patients from 5 kindreds with low TRECs that presented overlapping microdeletions on chromosome 2p11.2 spanning the *FOXI3* gene were described [58]. *FOXI3* haploinsufficiency was postulated to be the cause of T-cell lymphopenia in these patients. FOXI3 is involved in the same pathway critical for thymus development in which also TBX1 and FOXN1 are key players, and patients with haploinsufficiency in all these genes share similar phenotypes [55, 56, 59, 60]. Additionally, heterozygous *Foxi3*-mutant mice show a smaller thymus [58]. We recently confirmed that *FOXI3* haploinsufficiency is another cause of T-cell lymphopenia at birth [61]. We reported two unrelated subjects with low TREC levels at birth and T-cell lymphopenia, demonstrated heterozygous loss-of-function variants in the *FOXI3* gene in both of them. We confirmed that the T-cell lymphopenia was not caused by an intrinsic defect of hematopoietic cells, by using an *in vitro* T-cell differentiation assay based on an artificial thymic organoid (ATO) platform [62, 63]. Indeed, $CD34^+$ cells from the peripheral blood of one of the subjects carrying the *FOXI3* variant were able to efficiently differentiate into mature T cells *in vitro*, with kinetics and absolute numbers comparable to those of $CD34^+$ cells isolated from a normal control. T-cell lymphopenia in subjects carrying *FOXI3* variants may improve over time in which case no definitive therapy is required); however, because of the low number of patients identified so far, the natural history of the disease and its severity remain to be fully defined.

This is an important example of how critical is for the timely and correct management of patients to understand whether subjects presenting with severe and persistent T cell lymphopenia and low TREC carry genetic defects affecting the hematopoietic cells or the thymic stromal cells. Discriminating the hematopoietic-intrinsic versus -extrinsic nature of the defect would indeed allow to choose the most effective treatment for the patient. In a T-cell lymphopenic infant with a heterozygous *FOXN1* variant of uncertain significance, the ATO assay could help by indicating in a reasonably timely manner whether the T-cell

lymphopenia is caused by a hematopoietic or a thymic defect. During the past years, many groups have worked on the development of efficient *in vitro* assays for T cell differentiation, taking advantage of the Notch ligand signaling, mediated by stromal cell engineered to express DLL4 or DLL1 or through binding of these ligands to cell culture plates [64, 65]. Recently Seet and colleagues [63] published a serum-free 3D artificial thymus organoid (ATO) system, generated by aggregating $CD34^+$ cells with a murine stromal cell line expressing the Notch ligands DLL1 or DLL4, that could efficiently and reproducibly generate mature $TCR\alpha\beta^+$ $CD3^+$ T cells in less than two months. We and others [62, 66] tested the ATO system using $CD34^+$ cells isolated from peripheral blood or bone marrow of patients carrying known mutations in genes causing T-cell lymphopenia of different severity and could establish that this system was able to reliably discriminate the hematopoietic-intrinsic or - extrinsic nature of the defect and to recapitulate the block in the T cell development in case of hematopoietic autonomous defects. Furthermore, the ATO system demonstrated that mouse models might not faithfully recapitulate the equivalent human conditions, as in the case of *RAG* deficiency. Indeed, in *Rag*-deficient mice, T cell development is blocked at double-negative 3 (DN3 stage), while $CD34^+$ cells from *RAG*-deficient patients were able to differentiate up to double positive (DP) cells when cultured in the ATO system [62, 66]. Notably, the only case in which the ATO system could not reproduce the hematopoietic cell-autonomous block in T cell development was represented by adenosine deaminase (ADA) deficiency. $CD34^+$ cells from these patients are able to differentiate into mature $TCR\alpha\beta^+$ $CD3^+$ cells in the ATO platform, likely because the stromal cell line included in this system produces ADA, allowing for cross-correction of the hematopoietic cell defect. We subsequently used the ATO system to confirm the hematopoietic or thymic stromal nature of diseases with novel genetic causes, such as *POLD1* [67], *SASH3* [68] and *FOXI3* deficiency [61]. We believe that this system provides a powerful assay that can quickly guide decisions on clinical interventions in cases of infants with life-threatening severe T-cell lymphopenia of unknown genetic etiology.

## 4. Novel tools to model thymic epithelial cell development

Although the ATO system has proved to be very useful in determining whether the cause of T-cell lymphopenia might be hematopoietic or thymic stromal-based, this system can only be used to study the precise block in development in specific hematopoietic intrinsic defects. $CD34^+$ cells of patients with thymic defects are able to efficiently differentiate into mature T cells when cultured in the ATO system, in which T-cell differentiation is supported by the murine stromal cell line expressing the Notch ligand and by the cytokines and supplements provided in the culture medium. Thus, in order to model defects affecting thymic stromal cells, alternative assays aimed at reproducing TEC development need to be generated. In the past years, knowledge on the signaling pathways driving thymus development has increased considerably and has led to the generation of many different protocols for the induction of TEC progenitor cells (TEPs) and TECs starting from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs).

One of the first successful protocols was published by Lai and Jin [69], who were able to generate TEPs *in vitro* starting from murine ESCs using a combination of FGF7, FGF10, BMP4 and EGF. They cultured mESC cells in 3D and 2D conditions, and obtained the

highest frequencies (about 25%) and absolute numbers of EpCAM$^+$ cells in 2D cultures in the presence of all the factors. These cells expressed in large majority (between 68 and 82%) both cTEC and mTEC markers, such as Keratin 8 (K8) and Keratin 5 (K5), and importantly also showed expression of TEC genes *Pax1*, *Pax9* and *Foxn1*, although at levels lower than those found in EpCAM$^+$ cells isolated from mouse embryonic thymi. Purified mESC-derived EpCAM$^+$ cells were able to differentiate into mature cTECs and mTECs when reaggregated with CD4$^-$CD8$^-$ CD45$^+$ thymocytes and transplanted *in vivo* under the kidney capsule of syngeneic mice. Grafts harvested 6 weeks after transplants showed evidence of thymopoietic activity, since they contained CD4 and CD8 DP and single positive (SP) cells. Interestingly, the authors showed that mESC-derived EpCAM$^+$ cells, when injected in the thymus of lethally irradiated mice prior to bone marrow transplant, were capable of increasing thymocyte reconstitution and peripheral naïve T cell numbers.

A year later, another report from Inami and colleagues [70] published an improved protocol for the generation of TEPs starting from human ESCs and iPSCs. The most significant change they apported to the protocol of Lai and Jin was the addition of an initial step of differentiation of 4 days with Activin A and Lithium Chloride (LiCl) to induce definitive endoderm phenotype, prior to adding the factors used in the previous protocol (FGF7, FGF8, FGF10 and BMP4) for the induction of TEP. Finally, after 12 days of culture, they added an extra step of 4 days, involving the use of RANKL, and aimed at promoting further maturation of TEP. The addition of RANKL greatly increased the expression of the TEC genes *Pax1*, *K5* and *Foxn1* and also showed induction of low levels of Aire, indicating that the exposure of TEPs to RANKL induced their further maturation towards the mTEC lineage. Unfortunately, this study did not provide evidence for the ability of the TECs obtained through this protocol to regenerate a thymus microenvironment or to possess thymopoietic activity, *in vitro* or *in vivo*.

Another interesting approach to readily identify and select hESC-derived TEP was published a few years later by Soh and colleagues [71]; this approach entailed the generation of FOXN1-GFP reporter hESC lines, in order to readily identify FOXN1 expressing cells by using GFP. The authors were able to efficiently generate TECs from these hESC reporter lines by culturing them as embryoid bodies and adding Activin A for the first 4 days, and then FGF7 starting at day 14, and replenishing it once a week up to 35 days. Starting from day 12, differentiation cultures showed a progressive upregulation of pharyngeal pouch markers (HOXA3 and PAX9) and epithelial cell markers (IVL and FOXN1), while endodermal markers (SOX17 and FOXA2) were downregulated. However, functional assessment of FOXN1$^+$ hESC-derived TEP, performed co-culturing them with ProT cells did not provide evidence of thymopoietic activity, suggesting that the TEPs obtained through this protocol may be functionally inadequate to promote T cell differentiation.

Great advancement in the protocols for the generation of TEPs were made in two reports published by Parent and colleagues [72] and Sun and colleagues [73]. They were able to develop multi-step protocols during which the expression of markers characteristic of the several stages of differentiation from hESC to TEPs, mimicking the *in vivo* thymus organogenesis, could be monitored over time.

Both protocols showed that the introduction of Retinoic Acid (RA), which was previously shown to be a key molecule in the early formation of pharyngeal pouches [6], was critical for the anteriorization of the definitive endoderm and the induction of the markers of the third Pharyngeal Pouch Endoderm (PPE), such as *HOXA3*, *TBX1* and *EYA1*. Further directed differentiation from PPE to TEP was achieved by Sun and colleagues by using BMP4 and WNT3, while Parent and colleagues in addition to these factors also used the Hedgehog inhibitor Cyclopamine (CPM) and FGF8. Both protocols showed efficient generation of TEPs, which could further mature into functional TECs able to support T cell differentiation upon transplantation into athymic mice.

Importantly, the protocol developed by Parent and colleagues was shown to be efficacious in modeling the effects of mutations in two genes recently discovered to cause thymic stromal cell defects, *EXTL3* and *PAX1* [74, 75]. EXTL3 is a glycosyltransferase involved in the synthesis of heparan sulfate, and homozygous missense variants in this gene were demonstrated to cause T-cell lymphopenia, severe skeletal dysplasia, and developmental delay [74]. Volpi and colleagues generated iPSC from fibroblasts obtained from a patient carrying a homozygous variant in *EXTL3* and evaluated the ability of these cells to generate TEP, in comparison to an iPSC line generated from a normal control. They could demonstrate that TEP differentiated from the *EXTL3*-mutated patient's iPSC had a decreased expression of TEC-specific genes, such as *FOXN1*, *K5* and *EYA1*, while retained higher expression of *SOX17*, a gene that in the control line reached a peak at the DE stage and was subsequently downregulated [74]. A similar analysis was performed in TEP differentiated from iPSC lines generated from patients carrying *PAX1* mutations [75]. PAX1 is a transcription factor that plays a critical role during embryogenesis, as it is expressed in the pharyngeal pouches from which the thymus, tonsils, parathyroid glands, thyroid, and middle ear derive [9]. Mutations in this gene cause a rare syndrome called otofaciocervical syndrome type 2 (OTFCS2), characterized by facial dysmorphism, ear anomalies and hearing loss, skeletal malformations, mild intellectual disability and severe T-cell lymphopenia [75, 76]. Gene expression profile of TEPs obtained from *PAX1*-mutant patients showed that several genes were decreased in comparison to TEPs obtained from normal donors, including genes crucial for TEC development such as *FOXN1*, *TP63* and *BMP4*, but also genes involved in skeletal, cartilage, pharyngeal, neural crest and ear development, that would account for the broad range of malformations presented by patients carrying *PAX1* mutations [75].

Additional approaches to improve efficiency of TEP generation took advantage of proof-of-principle studies showing that Foxn1 over-expression could alone induce reprogramming of murine fibroblasts into functional TEC, able to support T cell differentiation both *in vitro* and *in vivo* [77] and that culture of ESCs with recombinant HOXA3 and FOXN1 would significantly enhance TEP induction [78]. Based on these evidence, Otsuka and colleagues [79] described a protocol of TEP differentiation using an iPSCs line engineered to constitutively express Foxn1 and demonstrated that Foxn1 expression enhanced the differentiation of cells expressing TEC markers, along with the up-regulation of the endogenous *Foxn1* gene. At about the same time, a report from Chhatta and colleagues [80] showed that transduction of a lentiviral vector encoding for a codon-optimized *Foxn1* gene in PPE cells obtained from human iPSCs, by using a protocol adapted from Parent and

colleagues [72], could significantly improve the functionality of the TEP generated. Both reports showed that forced expression of Foxn1 could significantly enhance TEP ability to support T cell differentiation and induce tolerance [79, 80].

Further optimization of the protocols for TEP differentiation were recently published by Ramos and colleagues [81] and Gras-Peña and colleagues [82]. Both reports introduced the use of Sonic Hedgehog (SHH) activation during the step of pharyngeal endoderm induction, and this resulted in increased expression of *PAX9*, *PAX1*, and *TBX1* genes. Interestingly, Gras-Peña and colleagues introduced in their protocol the use of Noggin, a BMP4 antagonist, between the pharyngeal endoderm and the TEP induction steps and prior to adding again BMP4. The introduction of Noggin increased significantly the expression of both *FOXN1* and *PAX9* in TEP at the end of the differentiation [82]. Remarkably, the cells obtained at the end of this protocol, expressed many thymic marker genes, including *FOXN1*, *PAX1* and *AIRE*, at levels comparable to those found in fetal thymus, and when transplanted *in vivo* in immunodeficient mice, mixed with human thymic mesenchymal cells, could further mature and transiently support human T-cell development, but were not able to organize in a thymus-like structure [82]. In a crucial experiment performed by Ramos and colleagues, at the end of their TEP differentiation protocol, they reaggregated these cells and transplanted them *in vivo* in athymic nude mice. Fourteen to nineteen weeks after transplant, they harvested the thymic grafts and performed bulk and scRNAseq analyses. They could demonstrate that TEC differentiate *in vivo* from TEP, and that their transcription profile is similar to that of primary post-natal TEC. However, even with this improved protocol they could not retrieve clusters of more mature cTECs and mTECs, indicating that there is still room for improvement in TEP differentiation protocols. Nonetheless, scRNAseq analysis of the TECs isolated from the thymic grafts suggested a previously unrecognized role for NOTCH pathway in human TEC development, in addition to providing a list of target genes that could be important in human thymus development and could be exploited to further improve protocols for the generation of TEC *in vitro*.

A critical improvement in TEC generation could arise from the use of 3D culture systems. Indeed, it is known that the 3D structure is fundamental for the maintenance and functionality of TEC [83], and Zeleniak and colleagues [84] recently demonstrated how performing TEP generation from iPSC maintained in 3D alginate capsules led to increased expression of TEC markers. More importantly, when these cells were introduced in decellularized murine thymic scaffold, together with human $CD34^+$ hematopoietic progenitor cells, they were able to mature further into both cTECs and mTECs and could support generation of mature $CD4^+$ and $CD8^+$ T cells, both *in vitro* and *in vivo*.

In summary, the results obtained on TEP differentiation over the years have shown that much progress has been made in generating cells that can now express TEC gene markers at levels comparable to primary human TEC, and that these cells can be used to recapitulate gene defects affecting TEC development. However, further improvement in the differentiation protocols is still needed in order to achieve maturation of these cells *in vitro*, able to recapitulate the complexity of mature cTEC and mTEC subsets that constitute the human thymus. Attaining this goal would not only allow to model *in vitro* defects of thymus development affecting later stages of TEC maturation (eg. *AIRE* deficiency) but

could also generate TEC that could be used in *in vitro* T cell differentiation assays, such as the ATO system, instead of murine stromal cell lines, thus allowing the generation of thymic organoids reproducing more thoroughly the human thymus.

## 5. Conclusions and future prospective

The extensive new knowledge achieved in the recent years in molecular and cellular features of the human thymus and the consequent better understanding of the inter-cellular cross-talk among cell subsets, in terms of interactions and spatial localization, will provide critical information for the development of *in vitro* models able to faithfully recapitulate the human thymus microenvironment. It can be anticipated that this will allow not only a more efficient generation of T cells *in vitro*, but also provide better models to assess the impact of novel gene defects on thymus development and function. Ultimately, all the knowledge that would be achieved through these improved models will provide crucial novel insights into thymic development and function that could be exploited to develop future strategies of thymus engineering for the treatment of thymic defects.

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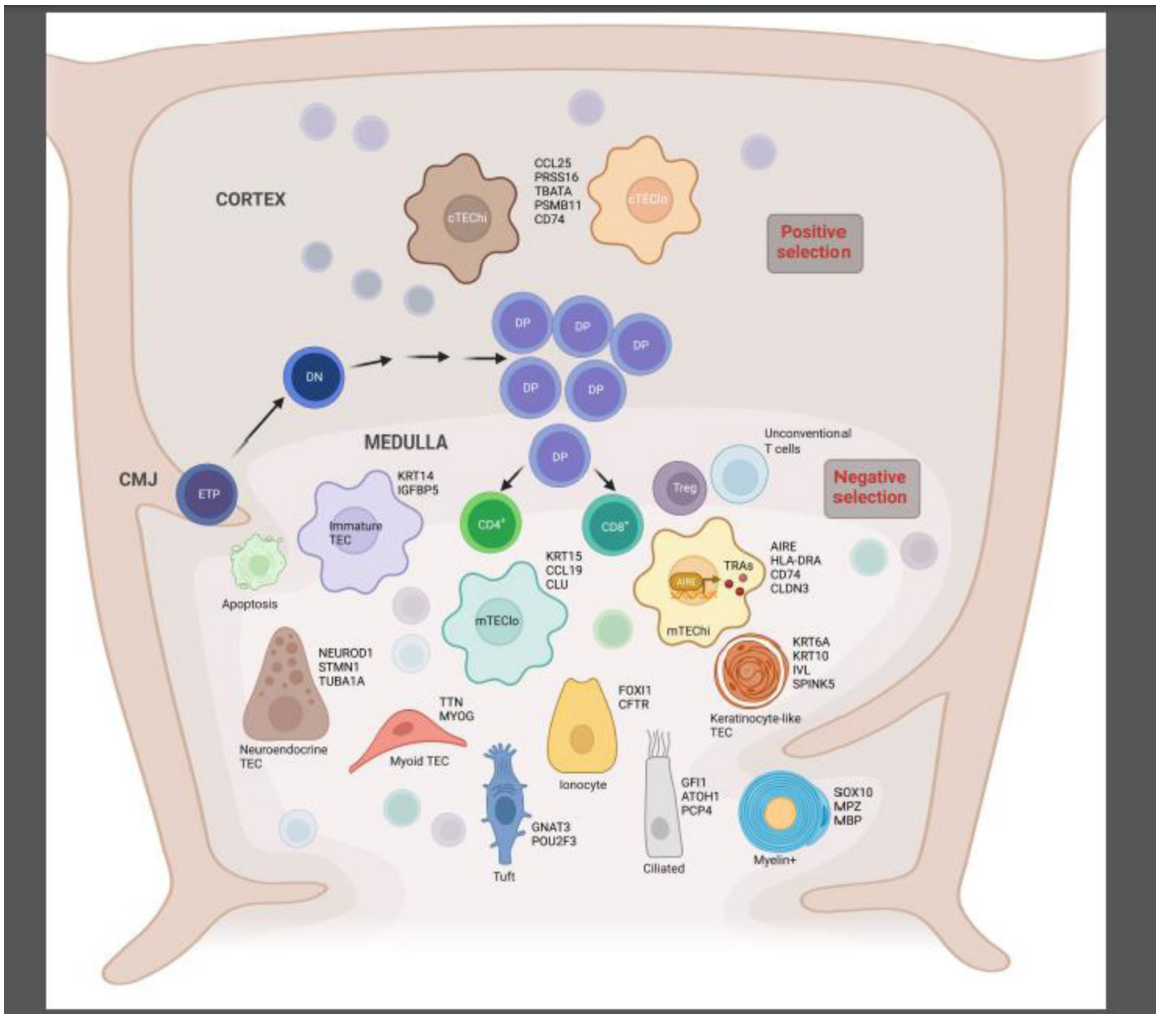


**Figure 1: Subsets of thymic epithelial cells described in human thymus and their representative markers**

Simplified schematic representation of T cell development in human thymus, indicating the early stages of development from the entrance of bone marrow-derived early thymic progenitors (ETP) at the cortico-medullary junction (CMJ), through the different steps of their positive selection in the cortex, from $CD4^{-}$ $CD8^{-}$ double negative (DN) to $CD4^{+}$ $CD8^{+}$ double positive (DP). The thymocytes then move to the medulla where they complete their maturation, undergo negative selection to eliminate self-reactive T cell specificities and give rise to the different subsets of mature T cells: $CD4^{+}$, $CD8^{+}$, T regulatory cells (Treg) and unconventional T cells. Cortical thymic epithelial cell (cTEC) and medullary TEC (mTEC) subsets recently described in human thymus are represented here. For each epithelial cell subset, some of the specific markers are listed.

**Table 1:**

Congenital Thymus Disorders

| Disease | Genetic defect | Inheritance | OMIM | Immune cells | Additional features |
|---|---|---|---|---|---|
| **DiGeorge/velocardio-facial syndrome Chromosome 22q11.2 deletion syndrome (22q11.2DS)** | *Large deletion (1.5–3Mb) typically in chromosome 22 (TBX1)* | AD | 602054 | T cells are decreased or normal 5% have low TRECs at NBS<br>B cells are normal | CHD<br>Hypoparathyroidism<br>Velopalatal insufficiency<br>Facial dysmorphisms<br>Intellectual disability [59, 85–92] |
| **DiGeorge/velocardio-facial syndrome** | Unknown | Sporadic | | T cells are decreased or normal<br>B cells are normal | |
| **TBX1 deficiency** | *TBX1* | AD | 602054 | T cells are decreased or normal<br>In some cases low TRECs at NBS<br>B cells are normal | |
| **TBX2 deficiency** | *TBX2* | AD | | T cells are decreased or normal | CHD<br>Craniofacial dysmorphisms<br>Developmental defects<br>Skeletal malformations<br>Endocrine abnormalities [93, 94] |
| **CHARGE syndrome** | *CHD7*<br>*SEMA3E*<br>Unknown | AD<br>AD | 608892<br>608166 | T cells are decreased or normal<br>In some cases low TRECs at NBS<br>B cells are normal | CHD<br>Coloboma of the eye<br>Choanal atresia<br>Intellectual disability<br>Genital and ear anomalies [95–98] |
| **Winged helix nude FOXN1 deficiency** | *FOXN1* | AR | 601705 | T cells are decreased<br>B cells are normal | Athymia<br>Congenital alopecia<br>Nail dystrophy [99–103] |
| **FOXN1 haploinsufficiency** | *FOXN1* | AD | 600838 | Severe T cell lymphopenia at birth<br>T cell numbers normalized in adults<br>B cell numbers can be normal or low | Recurrent respiratory tract infections<br>Skin involvement (eczema, dermatitis)<br>Nail dystrophy [55, 57] |
| **Chromosome 10p13-p14 deletion syndrome (10p13-p14DS)** | *Del10p13-p14* | AD | 601362 | T cells are decreased<br>B cells are normal | CHD in some cases<br>Hypoparathyroidism<br>Renal disease<br>Deafness<br>Growth retardation<br>Facial dysmorphisms [104–107] |
| **Chromosome 11q deletion syndrome (Jacobsen syndrome)** | *11q23del* | AD | 147791 | T cells are decreased<br>B cells are decreased<br>Immunoglobulins and antibody responses are decreased<br>NK cells are decreased | Recurrent respiratory tract infections<br>Multiple warts<br>Facial dysmorphism<br>Growth retardation [108] |
| **Chromosome 2p11.2 microdeletion** | *2p11.2del (FOXI3)* | AD | | T cells are decreased | Transient hypocalcemia<br>Asymmetric crying face [58] |
| **FOXI3 haploinsufficiency** | *FOXI3* | AD | | T cells are decreased | |
| **Immunoskeletal dysplasia with neurodevelopmental abnormalities (EXTL3 deficiency)** | *EXTL3* | AR | 617425 | T cells are decreased<br>B cells are normal<br>Eosinophilia | Short stature<br>Cervical spinal stenosis<br>Neurodevelopmental defects [74, 109, 110] |
| **Immunodeficiency with multiple intestinal atresias** | *TTC7A* | AR | 609332 | Variable T cell numbers<br>May have low TRECs at NBS<br>B cell numbers are normal or low<br>Hypogammaglobulinemia | Recurrent infections<br>Multiple intestinal atresias [111–114] |
| **Otofaciocervical syndrome type 2 (OTFCS2)** | *PAX1* | AR | 615560 | Severe T cell lymphopenia<br>Low TRECs<br>B cells are normal | Athymia<br>Ear abnormalities |

| Disease | Genetic defect | Inheritance | OMIM | Immune cells | Additional features |
|---|---|---|---|---|---|
| | | | | | Winged scapulae, abnormal clavicles [75, 76] |
| **Autoimmune Polyendrocrinopathy with Candidiasis and Ectodermal Dystrophy (APECED, APS-1)** | *AIRE* | AR or AD | 240300 | Normal T and B cell numbers | Multiple autoimmune manifestations<br>Dental enamel hypoplasia<br>Alopecia areata<br>Enteropathy<br>Pernicious anemia<br>Chronic mucocutaneous candidiasis [115–117] |

AD: autosomal dominant; AR: autosomal recessive; CHD: congenital heart defect; NBS: newborn screening; TREC: T cell receptor excision circles