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Review Article

De novo Thymus Reconstitution: The Rising of Cell-based Strategies

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Abstract

The thymus plays a crucial role in the generation of functional T cells, which are essential in adaptive immune responses. However, the involution, dysfunction, and even absence of the thymus induced by various factors such as aging, heredity, tumor, infection, and surgical removal, greatly impair or completely deprive the normal functions of the thymus and has been threatening the health of countless patients. Thus, reconstituting the thymus in these patients is remarkably necessary and urgent, in which thymus transplantation is viable, but the rare donors and related complications dramatically limit its clinical application. Other exogenous regeneration therapies, like sex steroid inhibition and cytokines treatments, usually have nonspecific, limited, and transient outcomes. By comparison, *de novo* cell-based strategies to reconstitute the thymus may be more feasible, which can generate fully functional thymuses usually using autologous cells without the dependence on limited thymus donors, thus avoiding transplant donor shortage as well as related complications, and overcome the defects of exogenous regeneration therapies. In this review, we summarized the progression in this field, including the generation of functional thymic epithelial cells (TECs), the clonogenic culture of TECs, stem cell treatment, and the construction of thymic organoids, to provide a global perspective for cell-based *de novo* thymus reconstitution.

Keywords

Thymus Reconstitution, Thymic Epithelial Cells, Thymic Organoids, Functional T Cells

1. Introduction

The thymus plays a crucial role in the maturity of functional T cells and adaptive immune responses. It is well known that hematopoietic precursor cells must undergo positive and negative selection by interacting with thymic epithelial cells (TECs) in the thymus to generate mature T cell repertoire, which further reaches corresponding sites to serve immunologic functions [1]. However, the involution, dysfunction, and even absence of the thymus induced by various factors (such as aging, heredity, tumor, infection, and surgical removal) greatly impair or completely abolish the normal functions of the thymus [2], leading to severe adaptive immune deficiency or autoimmunity that has been threatening countless human lives. Physiological aging of the human thymus begins after the first year of life, its size reduces at a rate of 3% per year until meddle age, and continue to reduce thereafter at a rate less than 1% each year [3], which is ac-

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companied by the increasingly deteriorating function of the thymus [4]. Inborn thymus defects like DiGeorge syndrome caused by chromosome 22q11.2 deletion, result in thymic hypoplasia or athymia, and T cell-mediated cellular immunodeficiency [5]. Tumors like thymomas, derived from TECs, may cause autoimmune diseases because of the deficiency of normal functional TECs [6]. Meanwhile, infections by bacteria, viruses, parasites, or fungus may impair TECs and alter extracellular matrix, leading to the damage of thymic function [7]. In addition, the thymus of new-born children with congenital heart disease is often removed for better access to the heart, however, this results in their susceptibility to various infections because of the thymus absence [8].

Reconstitution of the thymus in patients with thymic defects is remarkably necessary and urgent, in which thymus transplantation is optional, but the rare donors and almost inevitable complications dramatically limit its clinical application [9]. Meanwhile, exogenous treatments, including cytokines (insulin growth factor (IGF), keratinocyte growth factor (KGF), IL-15, IL-7, IL-21, and IL-22) [10-14] and sex steroid inhibition [15-17], have been reported to improve thymus involution to a certain degree. However, the effects of these therapies are nonspecific, limited, and transient [18]. For example, IL-22 was reported to increase the proliferation and survival of TECs in mice thymus, but its effects are limited to a damaged state [12]; KGF has shown to promote the proliferation and differentiation of TECs in mice, however, it failed to improve thymic function in oral mucositis patients [19]. In addition, GnRH, one of androgen receptor blockers, has shown to improve the functions of TECs and the production of naïve T cells in men with prostate cancer [16]. Luteinizing hormone receptor agonists, such as Lupron, have been reported to increase CD4 T cells and promote the expression of DLL4 in murine studies [15, 17]. However, the effectiveness of these non-targeted sex hormone suppression treatments in human is probably systemic and needs more investigations to verify [18, 19]. By comparison, cell-based *de novo* reconstitution of the thymus may be more feasible, which can produce functional thymuses usually using autologous cells without the dependence on thymus donors, thus avoiding transplant donor shortage as well as related complications, and overcome the defects of exogenous regeneration treatments. In this review, we summarized the progression in cell-based *de novo* thymus reconstitution, including the generation of functional TECs, the clonogenic culture of TECs, stem cell treatment, and the construction of thymic organoids, to provide a global perspective.

2. Generation of TECs

TECs play crucial roles in the development and maturation of functional T cell repertoire, which are divided into cortex TECs (cTECs) and medullary TECs (mTECs). The main functions of cTECs include T cell lineage specification and positive selection of immature T cells, while mTEC is mainly responsible for the autoimmune tolerance and single positive selection to generate mature T cell repertoire [20]. Thus, generating functional TECs is the basic strategy to reconstitute thymic functions (Table 1).

2.1. TEC Cell Line Construction

Cell lines are very useful tools in biomedical research. Beaudette-Zlatanova BC *et al.* infected human primary TECs with amphotropic retrovirus (PA317 LXSN-16E6E7) to establish immortalized TEC cell lines, in which a cell line highly expressing murine Delta-like 1 (TEC-Dl1) was isolated. Moreover, TEC-Dl1 cell line supported the generation of $CD4^+/CD8^+$ T cell from human cord blood and bone marrow hemopoietic progenitor cells (HPCs) *in vitro* [21]. Besides, Chen P *et al*. established thymic epithelial stromal cell lines (TSCs) from C57BL/6 E14.5 fetal thymus to construct TEPC lines, which express autoimmune regulator (Aire) and Aire-dependent tissue-restricted antigens (TRAs) under RANK stimulation and differentiate into mTEC-like cells in the presence of NF-κB subunits RelB and p52 *in vitro*. In addition, TSCs can differentiate into mature TEC-like cells that can support some limited development of T cells *in vivo* after transplantation under the kidney capsules of nude mice [22].

2.2. TECs Derived from Stem Cells

The multi-differentiation potential of stem cells, including natural and induced pluripotent stem cells, makes them an ideal source for obtaining TECs. Lai L and Jin J successfully induced mouse embryonic stem cells (mESCs) into thymic epithelial progenitors (TEPs)-like cells, which can self-renew, develop into cTECs/mTECs, rebuild the typical thymic structure, and enhance thymocyte regeneration when transplanted *in vivo* [23]. The same research group further revealed that transplantation of mESC-derived TEPs efficiently established thymocyte chimerism and generated naive T cells in young and old recipients transplanted with allogeneic bone marrow. Moreover, the reconstituted immune system showed enhanced graft-versus-tumor activity and host tolerance, without graft-versus-host disease (GVHD) observed [24]. Meanwhile, Inami Y *et al*. reported that induced pluripotent stem cells (iPSCs) can be differentiated into mTECs in four steps using chemically defined conditions. The obtained mTECs highly expressed forkhead box N1 (FOXN1) and keratin 5 (K5), which align with normal mTECs [25]. Besides, Parent AV *et al*. directly induced the differentiation of human embryonic stem cells (hESCs) into TEPCs by precisely regulating TGFβ, BMP4, RA, Wnt, Shh, and FGF signaling *in vitro*. The obtained TEPCs further developed into functional TECs and supported functional T cell development *in vivo* after transplantation into thymus-deficient mice [26]. In addition, Sun X *et al*. directly

induced the differentiation of human ESCs (hESCs) into TEP-like cells (TEPLCs) by sequentially regulating Activin, retinoic acid, BMP, and WNT signals. The obtained TEPLCs expressed FOXN1 and could further develop into TEC-like cells expressing the functional thymic markers including MHC II and AIRE *in vivo* after transplantation, which supported mouse thymopoiesis in T-cell-deficient mice and promote human T cell generation in NOD/SCID mice engrafted with human hematopoietic stem cells (hHSCs) [27]. Finally, Su M *et al*. developed a novel protocol to efficiently induce the differentiation of hESCs into TEPs *in vitro* in the presence of BMP4, FGF7, FGF10, EGF, RA (BFFER), rFOXN1 and rHOXA3. After transplanted into mice, these induced TEPs developed into TECs, formed thymic structures, and supported the long-term generation of functional mouse T cells or a higher level of human T cell from co-transplanted human hematopoietic precursors [28].

2.3. TECs Generated by FOXN1 Reprograming

The transcription factor FOXN1 is critically required for the development of TECs [29]. Bredenkamp N *et al*. reported that enforced FOXN1 expression could reprogram primary mouse embryonic fibroblasts (MEFs) into functional TECs (iTECs), which supported efficient development of both $CD4^+$ and $CD8^+$ T cells *in vitro*, and established a complete, fully organized, and functional thymus that contained all of the TEC subtypes *in vivo* after transplantation into nude mice [30]. Recently, Ma Z *et al*. revealed that MEFs overexpressing FOXN1 upregulate markers of both cTEC and mTEC lineages. Meanwhile, it was demonstrated that promoting proliferation enhances iTEC generation, and Notch inhibition promotes mTEC differentiation. In addition, MHC-II expression of iTECs is lower than fetal TECs, which can be improved by co-culturing iTECs with fetal double-positive T-cells [31].

Table 1. *The strategies to generate TECs.*

Protocols	Effects	Mechanisms	References
Human primary TECs were infected with amphotropic retrovirus to establish immortalized TEC cell lines and a cell line highly expressing murine Delta-like 1 (TEC-Dl1) was isolated.	TEC-Dl1 cell line supported the generation of $CD4^+/CD8^+$ T cell from human cord blood and bone marrow HPCs *in vitro*.	Not mentioned.	[21]
TSCs were established from the fetal thymus to construct TEPC lines.	1. TEPC lines expressed Aire and TRAs under RANK stimulation and differentiated into mTEC-like cells *in vitro*; 2. TSCs can differentiate into mature TEC-like cells that can support some limited development of T cells *in vivo*.	Not mentioned.	[22]
mESCs were induced into TEP-like cells.	1. Obtained TEP-like cells can self-renew and develop into cTECs/mTECs; 2. TEP-like cells rebuild the normal thymic structure, and enhance thymocyte regeneration when transplanted *in vivo*.	Not mentioned.	[23]
mESCs were induced into TEP-like cells.	Transplantation of mESC-derived TEPs efficiently establishes thymocyte chimerism and generates naive T cells in both young and old recipients of allogeneic BM transplants.	Not mentioned.	[24]
iPSCs were induced to mTECs in four steps using chemically defined conditions.	The obtained mTECs highly expressed FOXN1 and K5, which are in line with normal mTECs.	Not mentioned.	[25]
hESCs were induced into TEPCs by precisely regulating TGFβ, BMP4, RA, Wnt, Shh, and FGF signaling *in vitro*.	The obtained TEPCs can further develop into functional TECs and support functional T cell development *in vivo*.	Not mentioned.	[26]
hESCs were directly induced into TEP-like cells (TEPLCs) by sequentially regulating Activin, retin-	The obtained TEPLCs expressed FOXN1 and could further develop into TEC-like cells *in vivo*, supporting mouse thymopoiesis and pro-	Not mentioned.	[27]

Protocols	Effects	Mechanisms	References
oic acid, BMP, and WNT signals.	moting human T cell generation in T-cell-deficient mice.		
hESCs were induced into TEPs *in vitro* in the presence of BMP4, FGF7, FGF10, EGF, RA (BFFER), rFOXN1 (100 ng/ml), and rHOXA3 (200 ng/ml).	The obtained TEPs can develop into TECs and form thymic structures, and support the long-term generation of functional T cells.	Not mentioned.	[28]
MEFs were reprogrammed into functional TECs by enforced FOXN1 expression.	The obtained TECs supported the efficient development of both $CD4^+$ and $CD8^+$ T cells *in vitro*, and established a complete and functional thymus that contained all of the TEC subtypes after *in vivo* transplantation.	Not mentioned.	[32]
MEFs were reprogrammed into functional TECs by enforced FOXN1 expression.	Mechanism research.	MEFs overexpressing FOXN1 upregulate markers of both cTEC and mTEC lineages. Meanwhile, it was demonstrated that promoting proliferation enhances iTEC generation, and Notch inhibition promotes mTEC differentiation.	[31]

3. The Clonogenic Culture of TECs

The clonogenic culture of TECs is the foundation of thymus reconstitution, which, however, is difficult, especially for those isolated from adult and elderly thymus. Thus, viable strategies for expanding TECs are essential. Based on the similar biological properties between the skin cells and TECs, Pinto S *et al*. cocultured isolated murine mTECs with human dermal fibroblasts to form a 3D organotypic coculture model, which preserves key biological and gene expression features of mTECs, and supports their proliferation and differentiation [33, 34]. Truong VX et al. prepared gelatin hydrogel crosslinked with a polyethylene glycol linker via the nitrile oxide-norbornene click reaction. They found that co-cultures of E14.5 GFP^+ TECs and fibroblast in these gels formed epithelial colonies after 7 days of incubation, though not as well as in Matrigel [35]. Sekai M *et al*. isolated TECs from mice thymus by enzymatic digestion and magnetic separation. The obtained TECs were then cocultured on an STO cell feeder layer, and the medium was refreshed every 3 days. After 3 days of cultivation, the medium is supplemented with 10 ng/mL EGF and 10^3 U/mL leukemia inhibitory factor (LIF). TEC colonies were visible around 3 days after cultivation and can be passaged after 7 to 10 days. In addition, it was found that TECs from the E14 embryo, newborn, and $Rag2^{-/-}$ thymus showed higher clonogenic activities than those from 4-week-old wild-type mice. Furthermore, the colonies derived from total TECs showed immature phenotypes and generated both mature cTECs and mTECs after being implanted *in vivo* [36, 37]. Recently, Adachi Y described the protocol for the serum-free culture of TECs isolated from newborn mice. The serum-free medium was prepared by basic medium (1: 1 mixture of Ca^{2+}-free DMEM and Ham's F-12 nutrient mix supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/ mL streptomycin) supplemented with 3 μg/mL recombinant human insulin (INS), 20 ng/mL recombinant human epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone (HC), and 10 ng/mL cholera toxin (CT). TECs were cultured in gelatin-coated wells and serum-free medium above to achieve clonogenic culture. The initial medium change was performed after 3 days, and half the medium volume was exchanged every 2 days after that. In the second or third week of culture, the TECs can be sub-cultured [38].

4. Stem Cell Treatment

The multiple differentiation potential of stem cells also makes them another option for directly participating in thymus reconstruction. Liu G *et al*. treated $Foxn1^{-/-}$ mice with human umbilical cord-derived mesenchymal stem cells (UC-MSCs), which integrated into thymus tissue and improved cortex-medulla architecture maturation of thymic epithelial cells. Meanwhile, enhanced export of mature T cells, including regulatory T cells in the peripheral blood, was observed, indicating improved thymic function [39]. Recently, Yang Z *et al*. treated aged rhesus monkeys with MSCs, and found that MSCs improved the structure and function of the thymus in elderly macaque monkeys by reg-

ulating DNA methylation. Specifically, the decreased methylation level of the transcription factor NGF resulted in upregulated expression of KRT17 and FOXJ1, leading to enhanced proliferation of TECs [40]. Meanwhile, *in vitro* study also revealed that UC-MSCs promote the proliferation of TECs by upregulating the expression of VEGFA, activating the PI3K-AKT signaling pathway, increasing the expression of CDK2 and CCNE, and decreasing the expression of P27 [41].

5. Thymic Organoids

Organoids are 3D cell cultures that contain some of the key properties of the organs they represent [42]. The thymus is a complex 3D organ composed of extracellular matrix scaffold and multiple types of cells, including TECs, TEPCs, thymic mesenchymal cells, endothelial cells, adipocytes, fibroblasts, etc. Thus, the thymic organoids need to mimic the refined structure of the natural thymus to achieve effective thymic reconstitution, and there are several existing strategies to constitute thymic organoids (Table 2).

5.1. Based on Scaffolds

5.1.1. Based on Decellularized Thymic Extracellular Matrix Scaffolds

Thymic extracellular matrix scaffolds are composed of extracellular matrix proteins, including collagen IV, fibronectin, and laminin, which provide perfect 3D attachment sites and supporting microenvironments for all types of thymus cells [43]. Fan Y *et al.* seeded $CD45^-$ TSCs (TECs and thymic fibroblasts, extracted from 2~3 week-old C57BL/6J mice) and lineage marker negative (Lin^-) progenitors into decellularized mouse thymus scaffolds to construct thymic organoids. The TECs in the organoids survived and maintained their normal characteristics and biomarkers. After being transplanted into nude mice, the organoid effectively promoted the homing and differentiation of lymphoid progenitor cells and supported thymus development. Meanwhile, the nude mice received transplantation efficiently rejected skin allografts, and generated specific cellular and humoral immune responses, as well as tolerance to MHC-compatible skin allografts, suggesting successfully reconstructed thymus functions *in vivo* [44]. Hun M *et al.* prepared decellularized mouse native thymic extracellular scaffolds. Then, E14.5 TECs and thymic fibroblasts were seeded into the scaffolds to establish thymic organoids. After being transplanted under the kidney capsule of nude mice, the organoid supported the formation of a complete thymic microenvironment, attracted hematopoietic progenitors, and produced mature T cells *in vivo* [45]. Besides, Tajima A *et al.* described a systematic approach to constructing thymic organoids based on mature TECs and decellularized thymus scaffolds, in which the thymus extracellular matrix scaffolds were prepared by repetitive freeze-thaw cycles and detergent-induced cell lysis, followed by the seeding of isolated mouse TECs. The reconstructed thymus organoids effectively promoted the homing of bone marrow-derived lymphocyte progenitors and supported the development of a fully functional T cell repertoire *in vivo* [46]. Campinoti S *et al.* constructed an anatomic phenocopy of the native thymus by introducing epithelial-mesenchymal hybrid cells capable of long-term expansion *in vitro* into decellularized mouse thymus scaffolds obtained by whole thymus perfusion. The thymic organoids supported mature T cell development *in vivo* after being transplanted into humanized immunodeficient mice [47]. In 2021, Asnaghi MA *et al.* developed 3D scaffolds based on acellular thymus tissue, which supported both *in vitro* and *in vivo* thymus development of fetal and adult TECs. In addition, the scaffold supported the long-term culture of adult TECs *in vitro* without losing the expression of FOXN1 [43]. Finally, Zeleniak A *et al.* induced human iPSC into TEPCs using a modified four-step protocol, which were seeded with thymic fibroblasts and human hematopoietic stem cells into mice thymic decellularized scaffolds to construct human thymus organoids. The human thymus organoids supported the *de novo* generation of mature human T cell populations. After being implanted into humanized immunodeficient mice, the organoids mediated cellular and humoral immune responses, including the production of potent pro-inflammatory responses after T cell receptor activation, suppression of allogeneic tumor xenografts, tolerance to MHC-compatible tumor xenografts, and promotion of effective Ig class switching [48, 49].

5.1.2. Based on Other Scaffolds

Except for natural thymic ECM scaffolds, other biocompatible scaffolds were also introduced to construct thymus organoids.

(i). Carbon Matrix Coated with Tantalum

In 2000, Poznansky MC *et al.* reported that 4~5 small thymus fragments from C57BL/6L mice were cultured on the surface of CellFoam disk, a porous material composed of reticulated carbon matrix coated with tantalum (a highly biocompatible metal) for 14 days. After that, $CD34^+$ or $AC133^+$ human hematopoietic progenitor cells (HSCs) (1×10^4) were introduced to the culture system. The system produced fully mature T cells with a broad repertoire after two weeks. It was also demonstrated that the 3D niches in the matrix, matrix size, and the number of input hematopoietic progenitor cells are critical to the output of T cells [50-52]. Meanwhile, Clark RA *et al.* arrayed human skin fibroblasts and keratinocytes onto a 3D tantalum-coated carbon matrix, which supported the generation of functional human T cells with T cell receptor excision circles, a diverse T cell repertoire, and tolerance to self-MHC from hematopoietic precursor cells. The skin cells express AIRE, FOXN1, and Hoxa3 transcription factors, and a panel of autoantigens, which may play mimic roles of

TECs [53].

(ii). Polymer Scaffolds

Palamaro L *et al*. seeded human epidermal keratinocytes and human dermal fibroblasts on the 3D poly ε-caprolactone scaffold to establish thymus organoids, which supported T cell differentiation from HSCs proved by the upregulation of T-cell lineage related markers including CD7, CD1a, Spi-B, PTCRA, and RAG2. However, no mature single positive T cell subsets were observed, probably due to improper 3D scaffold material [54]. Tajima A *et al*. developed a self-assembling 3D hydrogel artificial thymic extracellular matrix system based on amphiphilic EAK16-II oligopeptides and its histidinylated analogue EAKIIH6. After perfused with primary murine TECs, the thymic organoids enhanced the formation of cell aggregates *in vitro*, and effectively promoted the development of functional T cells *in vivo* after being transplanted into the athymic nude mice [55]. Besides, Bortolomai I *et al*. seeded *Oct4* gene-modified TECs into type I collagen scaffolds to establish thymic organoids. It was discovered that transient *Oct4* expression promoted the proliferation of TECs, but did not significantly change the cell lineage identity of adult TECs. In addition, *Oct4*-expressing TECs could grow in type I collagen scaffolds both *in vitro* and *in vivo*. However, *in vivo* experiments showed that the thymus organoid subcutaneously transplanted in nude mice was vascularized but could not support effective thymopoiesis for its limited survival time [56], suggesting that the type I collagen scaffold may not be suitable for constructing functional organoids probably due to its simple composition. Finally, Silva CS *et al*. immobilized fibronectin, laminin-2, and feeder cell-derived extracellular matrix at the surface of 3D porous and fibrous electrospun polycaprolactone meshes, and found that coating of these components increased the viability, proliferation, and deep migration of TECs, suggesting the potentials of extracellular matrix coated electrospun polycaprolactone meshes to support TEC culture and establishment of thymic organoids [57-59].

5.2. Based on Cell Aggregation and Air-liquid Interface Culture

Air-liquid interface culture allows the basal side of cells to contact the culture medium and the apical side to be exposed to air, thereby creating a microenvironment similar to that in *in vivo* tissues and promoting cell polarization and functional differentiation [60]. Seet CS *et al*. aggregated MS5 murine bone marrow stromal cell line transduced with human DLL1 (MS5-hDLL1) and HSPCs by centrifugation and deployed on a cell culture insert at the air-fluid interface to establish a serum-free, artificial thymic organoid (ATO) system. The ATO efficiently supports the generation of conventional human T cells with diverse TCR from all sources of HSPCs (human $CD34^+CD3^-$ HSPCs, human bone marrow progenitor subsets), and the generation of naïve TCR-engineered T cells *in vitro* [61]. Chhatta AR *et al*. induced TEPCs from iPSCs combined Foxn1 transduction using lentiviral vector, which retain a relatively immature phenotype indicated by co-expression of keratin 5 and 8. The thymic organoids were further prepared by aggregating induced TEPCs with mouse embryonic fibroblasts on semipermeable discs floating on medium. After transplanting these organoids under the kidney capsule of athymic $Foxn1^{-/-}$ nude mice, $CD4^+$ and $CD8^+$ single-positive T cells, and remarkably higher TCR diversity were discovered after 10 weeks. Meanwhile, the generated T cells can be activated by anti-CD3/CD28 antibodies *in vitro*, suggesting the generation of functional T cells [62]. Besides, Montel-Hagen A *et al*. modified their ATO system reported in 2017 [61]. Specifically, they induced human pluripotent stem cells (human embryonic stem cells or induced pluripotent stem cells) into mesoderm cells, then mixed them with MS5-hDLL4/hDLL1 cells, which were further aggregated by centrifuge and cultured at air-fluid interfaces. The modified ATO systems can continuously generate mature, functional T cells with a diverse TCR repertoire *in vitro* [63]. In addition, the same research group aggregated and cultured MS5-mDLL4 with bone marrow stem and progenitor cells (HSPCs) at air-fluid interfaces to establish a murine artificial thymic organoid (M-ATO) system *in vitro*. This ATO efficiently supported normal murine T cell development without losing the phenotypic and transcriptional features, even when initiated with one single HSC [64-66]. Gong M *et al*. constructed the OP9-DLL1 mouse bone marrow mesenchymal cell line, and extracted C57BL/6 mouse E13.5 fetal liver HSPCs and bone marrow HSPCs, which were then mixed, centrifuged, and aggregated before being cultured at an air-liquid interface. Within 40 days of induction culture, the thymus organoids exhibited a good state and gradually increased in volume. More importantly, it induced the differentiation of various sources of mouse HSPCs into T cells *in vitro* [67]. Gardner CL *et al*. established severe combined immune deficiency (SCID) patient skin fibroblast-derived and gene-repaired iPSCs, which were further induced into human embryonic mesodermal progenitors (hEMPs). Then, the obtained hEMPs were mixed with MS5-hDLL4 cells, centrifuged, and aggregated before being cultured at an air-liquid interface to construct thymic organoids. The thymic organoids can rescue the progressive T cell differentiation potential of *RAG2*-deficient cells to normal levels, with the generation of a diversified T cell repertoire [68]. Ramos SA *et al*. constructed isogenic stem cell-derived thymic organoids composed of TEPs, hematopoietic progenitor cells, and mesenchymal cells, which were all differentiated from the same hPSC line. The three types of cells were then centrifuged, aggregated, and cultured at an air-liquid interface to construct thymic organoids. The thymic organoids supported T cell development, expressed key markers of negative selection, including the autoimmune regulator (AIRE) protein, and facilitated regulatory T cell development [69]. Recently,

Hübscher T *et al*. established thymic organoids using TECs from the embryonic thymus, embryonic thymus mesenchymal cells, and mouse embryonic fibroblasts, which were mixed, pelleted, and cultured at the air-liquid interface. The organoids maintained thymus function *in vitro*, mediated physiological T-cell development upon reaggregation with T-cell progenitors, and showed epithelial diversity and the ability to attract T-cell progenitors after *in vivo* grafting [70].

5.3 Based on 3D Non-scaffold Culture

Except for the strategies above, non-scaffold 3D cell aggregation culture was also applied to construct thymic organoids. Seach N *et al*. describe methods for the efficient isolation and enrichment of TEC for downstream analyses as well as the reaggregation of embryonic progenitor epithelium to form a functional thymus graft under the kidney capsule *in vivo*. However, the effects of such thymic organoids were not mentioned [71]. Okabe M *et al*. induced TEC-like cells from C57BL/6 mouse embryonic fibroblast-derived iPSCs in a 3D spheroid culture system, which had comparable expression levels of FOXN1 with normal TECs and also expressed K5 and K8. The cells were not rejected when transplanted into recipient mice, and T cell development was normally supported. Meanwhile, the newly generated T cells showed immune tolerance to both donor and recipient MHC, and were able to reject allogeneic third-party skin grafts [72]. Besides, Gras-Pena R *et al*. differentiated hES-TEPs by mimicking developmental queues with FGF8, Retinoic Acid, Sonic Hedgehog, Noggin, and BMP4, which were then mixed with fetal swine thymus (SwTHY) grafts or human thymic mesenchymal cells, and implanted under the kidney capsules of immunodeficient mice that received human hematopoietic stem and progenitor and stem cells (hHPSCs) intravenously. Both strategies supported human T cell development [73]. Recently, Lim S *et al*. reported that they generated long-term (>2 years) expandable 3D TEC organoids from the adult mouse thymus. These clonal organoids can be induced to express *Foxn1* and generate functional cortical- and Aire-expressing medullary-like TECs upon RANK ligand and retinoic acid treatment. In addition, the TEC organoids support T cell development from immature thymocytes *in vitro* and *in vivo* upon transplantation into athymic nude mice [74].

Table 2. *The strategies to constitute thymic organoids.*

Cells	Scaffolds	Effects	Mechanisms	References
$CD45^-$ TECs and thymic fibroblasts (extracted from 2~3 week-old C57BL/6J mice), and Lin^- progenitors.	Decellularized mouse thymus scaffolds.	1. The organoids can effectively promote the homing and differentiation of lymphoid progenitor cells, and support thymus development *in vivo*; 2. The mice efficiently rejected skin allografts and generated specific cellular and humoral immune responses.	Not mentioned.	[44]
E14.5 TECs and E14.5 thymic fibroblasts.	Decellularized mouse native thymic extracellular scaffolds were prepared by CHAPSO.	1. The organoid supported the formation of a complete thymic microenvironment and produced mature T cells *in vivo*. 2. Decellularized thymic scaffolds promoted the differentiation of TEPCs *in vitro*.	Not mentioned.	[45]
mature TECs	Decellularized thymus scaffolds were prepared by repetitive freeze-thaw cycles and detergent-induced cell lysis.	The reconstructed thymus organoids effectively promoted the homing of bone marrow-derived lymphocyte progenitors and supported the development of a fully functional T cell repertoire.	Not mentioned.	[46]
Epithelial-mesenchymal hybrid cells capable of long-term expansion.	Decellularized mouse thymus scaffolds obtained by whole thymus perfusion.	The organoid supported mature T cell development *in vivo* after transplantation into humanized immunodeficient mice.	Not mentioned.	[47]
Fetal and adult TECs.	Base on acellular thymus tissue.	1. The scaffolds supported both *in vitro* and *in vivo* thymus development of fetal and adult TECs; 2. The scaffold supported the long-term	Not mentioned.	[43]

Cells	Scaffolds	Effects	Mechanisms	References
		culture of adult TECs *in vitro*.		
Human TEPCs and TECs were induced from iPSCs.	Decellularized mouse thymus scaffolds.	The organoids can mediate cellular and humoral immune responses, suppress the growth of allogeneic tumor xenografts, and promote effective Ig class switching.	Not mentioned.	[48, 49]
4~5 small thymus fragments from C57BL/6L mice.	CellFoam disk, a porous material composed of reticulated carbon matrix coated with tantalum.	1. The system produced fully mature T cells with a broad repertoire after two weeks; 2. The 3D niches in the matrix, matrix size, and the number of input hematopoietic progenitor cells are critical to the output of T cells.	Not mentioned.	[50-52]
Human skin fibroblasts and keratinocytes.	3D tantalum-coated carbon matrix.	The system supported generating functional human T cells from hematopoietic precursor cells.	It may be related to the expression of AIRE, FOXN1, and Hoxa3 transcription factors and a panel of autoantigens in skin cell cultures.	[53]
Human epidermal keratinocytes and human dermal fibroblasts.	3D poly ε-caprolactone scaffold.	1. The thymus organoid supported the differentiation of T-lineage committed cells from HSCs; 2. No mature single positive T cells were observed.	Not mentioned.	[54]
Primary murine TECs.	A self-assembling 3D hydrogel artificial thymic ECM system based on amphiphilic EAK16-II oligopeptides and its histidinylated analogue EAKIIH6.	1. The formation of TECs aggregates was enhanced *in vitro*; 2. The system effectively promoted the development of functional T cells *in vivo*.	Not mentioned.	[55]
Oct4 gene-modified TECs.	Type I collagen scaffolds.	1. *Oct4*-expressing TECs were able to grow in type I collagen scaffolds both *in vitro* and *in vivo*; 2. The thymus organoid transplanted subcutaneously in nude mice was vascularized but could not support thymopoiesis for its limited survival time.	Not mentioned.	[56]
mTECs	Fibronectin was immobilized at the surface of 3D porous and fibrous electrospun polycaprolactone meshes.	Fibronectin coating increased the cell viability, proliferation, production of ECM proteins, and deep migration of mTECs.	Not mentioned.	[57]
cTECs	Laminin-2 was immobilized at the surface of 3D porous and fibrous electrospun polycaprolactone meshes.	The presence of laminin-2 promoted the cell viability and proliferation of cTECs.	Not mentioned.	[58]
TECs	Feeder cell-derived extracellular matrix was immobilized at the surface of 3D porous and	ECM coating increased the cell viability, proliferation, and production of ECM proteins of TECs.	Not mentioned.	[59]

Cells	Scaffolds	Effects	Mechanisms	References
	fibrous electrospun polycaprolactone meshes.			
MS5-hDLL1 murine stromal cells.	MS5-hDLL1 murine stromal cells were aggregated with HSPCs by centrifugation and deployed on a cell culture insert at the air-fluid interface.	The ATO system supported highly efficient and reproducible *in vitro* differentiation and positive selection of conventional human T cells from all sources of HSPCs.	Not mentioned.	[61]
Mouse embryonic fibroblasts and TEPCs induced from iPSCs.	The thymic organoids were further prepared by aggregating induced TEPCs with mouse embryonic fibroblasts on semipermeable discs floating on the medium.	Functional $CD4^+$ and $CD8^+$ single-positive T cells, and remarkably higher TCR diversity were discovered after 10 weeks *in vivo*.	Not mentioned.	[62]
Human embryonic stem cell and induced pluripotent stem cell-derived mesoderm progenitors.	The cells were aggregated with HSPCs by centrifugation and deployed on a cell culture insert at the air-fluid interface.	The ATO system continuously induced functional T cells with a diverse T cell receptor repertoire.	Not mentioned.	[63]
Bone marrow stem and progenitor cells (HSPCs).	The cells were aggregated by centrifugation and deployed on a cell culture insert at the air-fluid interface.	This method efficiently recapitulates the phenotypic and transcriptional features of normal murine T cell development even when initiated with a single HSC.	Not mentioned.	[64-66]
OP9-DLL1 mouse bone marrow mesenchymal cell line, E13.5 fetal liver HSPCs, and bone marrow HSPCs from C57BL/6 mouse.	The cells were aggregated by centrifugation and deployed on a cell culture insert at the air-fluid interface.	1. Within 40 days of induction culture, this thymus organoid exhibited a good state and gradually increased in volume; 2. It induced the differentiation of various sources of mouse HSPCs into T cells *in vitro.*	Not mentioned.	[67]
1. Primary fibroblasts from patients were reprogrammed into iPSCs, and then edited to fix the RAG2 deficiency and induced into hEMPs; 2. MS5-hDLL4 cells.	hEMPs and MS5-hDLL4 cells were aggregated by centrifugation and deployed on a cell culture insert at the air-fluid interface.	The thymic organoids rescued the progressive T cell differentiation potential of *RAG2*-deficient cells to normal levels, with the generation of a diversified T cell repertoire.	Not mentioned.	[68]
Stem cell-derived thymic organoids consisting of TEPs, hematopoietic progenitor cells, and mesenchymal cells.	The cells were aggregated by centrifugation and deployed on a cell culture insert at the air-fluid interface.	The thymic organoids supported T cell development, expressed key markers of negative selection, including AIRE protein, and facilitated regulatory T cell development.	Not mentioned.	[69]
TECs from the embryonic thymus, embryonic thymus mesenchymal cells, and mouse embryonic fibroblasts.	The cells were aggregated by centrifugation and deployed on a cell culture insert at the air-fluid interface.	1. The thymic organoids maintained thymus functionality *in vitro*; 2. The organoids mediated physiological T-cell development, and attracted T-cell progenitors *in vivo.*	Not mentioned.	[70]
TEC and embryonic progenitor epithelium.	None	Not mentioned.	Not mentioned.	[71]
TEC-like cells were induced from C57BL/6	None	Functional T cell development was normally supported.	Not mentioned.	[72]

Cells	Scaffolds	Effects	Mechanisms	References
mouse embryonic fibroblast-derived iPSCs in a 3D spheroid culture system.				
hES-TEPs were differentiated, and then combined with fetal swine thymus grafts or human thymic mesenchymal cells.	None	Both strategies supported human T cell development *in vivo*.	Not mentioned.	[73]
TECs from adult mouse thymus were cultured to form expandable 3D TEC organoids.	None	1. The clonal organoids can be induced to express *Foxn1* and to generate functional cortical- and Aire-expressing medullary-like TECs; 2. TEC organoids support T cell development from immature thymocytes *in vitro* and *in vivo*.	Not mentioned.	[74]

6. Summary and Future Perspective

The thymus is essential to generate functional T cell repertoire, its dysfunction or absence causes serious deficiency in adaptive immune functions. Despite various existing approaches to reconstitute declined thymus functions such as thymus transplantation, cytokines, and sex steroid inhibition [19], functional TECs and thymic organoids that can survive for a long time *in vivo* are more viable strategies, especially the latter. Compared to non-scaffold thymic organoids, scaffold-dependent thymic organoids have more advantages, which provide sufficient 3D attachment sites for TECs and niches for the interaction between TECs and hematopoietic precursor cells. Among existing scaffold materials, thymus decellularized scaffold is promising because it can provide more naturally compatible microenvironments for TECs. However, like thymus donors, the source of human natural thymus decellularized scaffolds are extremely rare, feasible options include utilizing animal natural thymus decellularized scaffolds and producing human natural thymus decellularized scaffolds on a large scale using bio-fabrication technology like 3D bioprinting [75]. In addition, we also agree that functional thymus organoids need more than a few TECs, other cell types including TEPCs, thymic mesenchymal cells, endothelial cells, adipocytes, and fibroblasts, may play necessary roles in the maintenance of normal thymus structure and function [76]. Thus, more cell types may need to be introduced into the thymic organoids to achieve a fully functional thymic microenvironment.

Abbreviations

TECs	Thymic Epithelial Cells
IGF	Insulin Growth Factor
KGF	Keratinocyte Growth Factor
IL-15	Interleukin 15
IL-7	Interleukin 7
IL-21	Interleukin 21
IL-22	Interleukin 22
cTECs	Cortex TECs
mTECs	Medullary TECs
TSCs	Thymic Epithelial Stromal Cell Lines
HPCs	Hemopoietic Progenitor Cell
AIRE	Autoimmune Regulator
TRAs	Aire-dependent Tissue-restricted Antigens
mESCs	Mouse Embryonic Stem Cells
TEPs	Thymic Epithelial Progenitors
GVHD	Graft-Versus-Host Disease
FOXN1	Forkhead Box N1
K5	Keratin 5
K8	Keratin 8
hESCs	Human Embryonic Stem Cells
MEFs	Mouse Embryonic Fibroblasts
iTECs	Induced TECs
EGF	Epidermal Growth Factor
LIF	Leukemia Inhibitory Factor
HC	Hydrocortisone
CT	Cholera Toxin
UC-MSCs	Umbilical Cord-derived Mesenchymal Stem Cells
Lin-	Lineage Marker Negative
HSCs	Hematopoietic Stem Cells
MS5-hDLL1	MS5 Murine Bone Marrow Stromal Cell Line Transduced with Human DLL1
ATO	Artificial Thymic Organoid
SCID	Severe Combined Immune Deficiency
hEMPs	Human Embryonic Mesodermal Progenitors
hHPSCs	Human Hematopoietic Stem and Progenitor and Stem Cells

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Author Contributions

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Conflicts of Interest

The authors declare that the publication of this paper has no conflicts of interest.

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