Perspectives for Improvement of the Thymic Microenvironment through Manipulation of Thymic Epithelial Cells: A Mini-Review

Ailin Lepletier^{a,b} Ann P. Chidgey^b Wilson Savino^a

^aLaboratory of Thymus Research, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; ^bStem Cells and Immune Regeneration Laboratory, Department of Anatomy and Developmental Biology, Monash University, Melbourne, Vic., Australia

Key Words

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Abstract

Thymic involution during aging is a major reason for the decreased production of naive T cells and reduced immunity. Alterations within the thymic microenvironment, characterized by the loss of function of thymic epithelial cells (TECs) and fibro-adipogenetic transformation, seem to underlie this process, mainly through declining communication between thymic stromal cells and developing thymocytes. Specifically, the signaling mediated by cytokines and hormones secreted by TECs declines during aging. Many therapies based on the manipulation of growth factors and hormones have succeeded in partially recovering the lymphoid compartment and promoting thymic function. However, considering that aging-induced thymic involution is multifactorial, the thymic reestablishment achieved with treatments that target isolated pathways is incomplete and transitory. Here, we discuss the development of three novel approaches for potentially sustained thymic recovery: the induction of sustained forkhead box N1 expression, the activation of endogenous thymic epithelial progenitor cells (TEPCs), and the generation of TEPCs from pluripotent stem

cells. Combined approaches targeting both TECs and lymphoid cells will provide a potentially more effective strategy for sustained rejuvenation of the thymus.

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Introduction

The thymus is one of the first organs to degenerate postnatally, with thymic involution beginning early in life and significantly impacting on immune system aging [1]. It has been reported in all jawed vertebrates, which indicates that this is an evolutionary ancient and conserved event. Aging-induced thymus degeneration is associated with a reduction in the cellularity of thymic epithelial cells (TECs) and thymocyte subsets, fibro-adipogenetic transformation, loss of tissue structure, and abnormal architecture, resulting in a decline of naive T-cell export. The consequent changes in the peripheral T-cell compartment are believed to be, at least in part, responsible for the clinical signs of immunosenescence. Accordingly, elderly individuals show a decreased immunoresponsiveness, are more susceptible to new threats, and are less able to control infection [2]. Furthermore, immune recovery following cytotoxic treatments can be severely delayed in middle-aged and elderly patients, leading to increased morbidity and mortality due to opportunistic infections [3].

Table 1. Hallmarks of thymus atrophy during aging of humans and mice

Humans	Mice
Progressive decline of recent thymic emigrants [25]	Decreased thymic output [36]
Enlarged PVS due to replacement of lymphoid cells by adipocytes [71]	Increased number of adipocytes in thymic parenchyma expressing CCR5 and its ligands [72]
Increase in LIF, stem cell factor, M-CSF, and oncostatin M by TEC and adipocytes [29]	Decreased expression of Wnt4 as FoxN1 in TEC [58]
Decrease in IL-2, IL-9, IL-10, IL-13, and IL-14 production by TEC [29]	Reduction of cTEC and mTEC MHCII ^{hi} [12]
Increase in angiogenetic and endothelial factors by adipocytes [73]	Reduced proliferative TEC staining with Ki67 and increased level of apoptotic TECs [23]
	Differentiation of fibroblast into preadipocyte through the increase of PPAR γ [17]

The precise mechanisms that underlie age-dependent thymus atrophy are still unclear. The thymus relies on the supply of lymphoid precursors from the bone marrow (BM) for T-cell development. Therefore, intrinsic age-related alterations in the hematopoietic compartment, such as a reduced lymphoid-to-myeloid lineage ratio with age, likely play a role in exacerbating thymic involution [4]. However, it appears that the thymic microenvironment itself is also responsible for influencing many of the defects exhibited by the T-cell compartment [5, 6].

Accordingly, studies on young adults, thymectomized during early childhood, demonstrate that despite their young age, some patients exhibit profound long-lasting perturbations, typically associated with advanced aging (i.e. >75 years old), as decreased T-cell count and very low naive T-cell frequency, reduced T-cell repertoire diversity, and increased numbers of highly differentiated memory T cells with shortened telomeres [7, 8]. In contrast to these studies, van Gent et al. [9] showed a normalization of the T-cell pool after 5 years of surgery. A slow recovery of the thymic tissue, detected by magnetic resonance imaging scans, was followed by the reestablishment of naive CD4⁺ and CD8⁺ T-cell counts and TRECs to normal ranges. However, the findings of this study were not uniformly confirmed.

Recent approaches to rejuvenate the immune system used in preclinical assays and in some clinical trials include modulation of the endocrine system such as sex steroid ablation or administration of growth factors such as growth hormone (GH), interleukin 7 (IL-7) and IL-22 [for a review, see 10]. Whilst these approaches impact on

improving thymopoiesis for the duration of treatment in preclinical models, it is unclear whether any parallel transient improvements in the TEC compartment are due to direct or secondary effects. Therefore, targeted interventions to rejuvenate the thymic epithelial compartment itself have recently emerged as a new generation of approaches.

Hallmarks of Thymic Involution: A Perspective from TEC-Related Mechanisms

Thymic senescence precedes that of other organs, resulting in an altered development and exportation of naive T cells to the periphery [11]. The mechanisms that underlie TEC-mediated transformations leading to the progressive decline in thymic function during aging are reviewed below, and findings in humans and mice are separately summarized in table 1.

Disruption of the Epithelial Tridimensional Network

Previous studies have demonstrated a clear loss in thymic epithelial tissue from as early as the first year of life, with >75% of the decline occurring up to middle age, followed by a gradual further decline [1]. TECs in aging mice have lower proliferative capacity and a higher rate of apoptosis compared with the values recorded in young animals [12]. As a consequence, there is a progressive increase in perivascular nonepithelial spaces, an associated breakdown of the defined cortical medullary junction, and a shift in the medullary-versus-cortical TEC ratio towards the cortical subset (cTEC) [12].

Fig. 1. Mechanisms underlying thymus atrophy during aging. The cross-talk between TECs and thymocytes is essential for thymus homeostasis. During aging, this communication is disrupted and the result is cellular depletion in both lymphoid (decrease in numbers of all thymocyte subsets) and epithelial compartments. Due to a depletion in TEC^{hi} , the ratio of $\text{TEC}^{\text{hi}}/\text{TEC}^{\text{int/lo}}$ gradually decreases with age. The impairment of TEC subsets seems to be at least partially related to their conversion to adipocytes, a phenomenon that seems to occur mainly in the thymic medulla.

An impairment in the cross-talk between TEC and developing thymocytes seems to be a detrimental contributing factor for epithelial structural disruption during aging. Several key inductive interactions between these cell types have been reported. The ratio of TECs with a high surface expression of major histocompatibility complex class II ($\text{MHCII}; \text{MHCII}^{\text{hi}}$) to TECs with an intermediate/low MHCII cell surface expression ($\text{MHCII}^{\text{int/lo}}$) gradually diminishes with age [12], as illustrated in figure 1. Thymocyte interaction is required for the upregulation of MHCII expression during ontogeny; therefore, this change may reflect the reduced levels of thymopoiesis. Medullary TEC (mTEC) formation, differentiation, and expansion require signaling from thymocytes through receptor activator of nuclear factor kappa-B ligand and CD40L [13].

The decreased availability of thymic epithelial niches for thymocyte progenitors in the involuted thymus or the deterioration in quality of these niches may account in part for reduced thymopoiesis in aging. Evidence supporting this notion comes from studies demonstrating that an increased TEC number produces a proportional increase in thymus size. The expression of cyclin D1 under a keratin 5 gene promoter in TEC results in a dramatic increase in thymus size and is sufficient to delay or prevent involution [14]. Using an embryo fusion chime-

ra-based approach, Jenkinson et al. [15] demonstrated that aging-associated thymus atrophy is related both to the restriction of thymus growth due to a reduction in the provision of growth factors as well as to a decrease in the proliferation of thymic epithelial progenitor cells (TEPCs). It seems that niche availability serves as a key determinant for thymus output and that restriction in the number and/or function of thymic microenvironmental niches is a critical cellular and structural component of thymic involution.

As thymic involution proceeds age-induced alterations in the BM, intrinsic defects in BM-derived hematopoietic stem cells (HSCs) and their capacity to generate high-quality lymphoid progenitors are likely to be contributing rather than initiating factors in thymic involution. Transferring young HSCs or early T-lineage progenitors (ETPs) from young mice into aged recipients alone does not restore thymic compartmental structure [16].

Process of Fibro-Adipogenesis

Thymic aging is associated with TEC decline and an increase in thymic fibroblast and adipocyte numbers. The adipocyte tissue accumulation is typically accompanied by an expansion of lipid-bearing cells within the thymic medulla [17] (fig. 1). Additionally, adipocyte infiltration

is observed in several thymic zones, including the perivascular nonepithelial space, the interlobular septa, the capsular region, and the subcapsular cortex [11]. Inhibition of thymic adipogenesis by calorie restriction has been shown to reduce age-related thymic involution and increase thymic function [17].

Although the mechanisms involved in adipogenesis along with thymic involution are not completely understood, there are indications that adipocyte accumulation during aging results from TEC conversion. Recent studies employing genetic fate mapping suggest that a given TEC subset can transdifferentiate into fibroblasts and adipocytes during aging, via the activation of an intrathymic epithelial-mesenchymal transition (EMT) process [18]. Actually, certain EMT cells that appear to commit to the adipocyte lineage in the aging thymus express peroxisome proliferator-activated receptor gamma (PPAR γ) and unilocular lipid droplets and appear to commit to the adipocyte lineage [18]. The elevation of PPAR γ and its upstream activator xanthine oxidoreductase (XOR) seems to drive thymus atrophy through the induction of ectopic adipogenesis. Accordingly, the specific inhibition of PPAR γ in thymic stromal cells induced reduction in EMT, preventing their adipogenetic transformation in an XOR-dependent mechanism and leading to an increased thymic export [17].

These data led us to conceive that the loss of TEC phenotype and the emergence of TEC-derived fibro-adipogenetic precursors may have direct implications in compromising thymopoiesis during aging.

Thymocyte Development Arrest

In the adult thymus, the fully developed tridimensional (3D) TEC network provides a unique microenvironment for hematopoietic progenitor cells, allowing thymic colonization as well as intrathymic T-cell migration and differentiation. In this respect, this 3D disposition, which is essential for thymocyte development, seems to be related to TEC induction of Delta-like (DL) 1 and DL4 and the consequent activation of Notch signaling in ETPs [19]. These progenitors are the earliest intrathymic precursors contained within the CD4CD8⁻ (DN) compartment and are characterized as DN1a/b cells, expressing the markers CD44⁺CD25⁻CD117⁺CD24^{int}. Aging mice have fewer ETP numbers, with reduced proliferation and differentiation potential. While ETP obtained from young mice are able to differentiate into all the stages of T-cell development when seeded into fetal thymic organ cultures [20], aging ETP show a reduction of T-cell differentiation activity, associated with an increased frequency of

apoptosis and a reduced expression of Ki67⁺ [21]. These age-induced alterations in ETP seem to result in part from intrinsic mechanisms, with the increased expression of the Ink4a tumor suppressor gene [22].

It has also been speculated that the reduction in ETP population observed in the aging mouse thymus is associated with impairment in the recruitment of BM-derived hematopoietic progenitor cells, mediated mainly by the CCL25 chemokine, produced by TEC and the expression of P-selectin by specialized thymic venules. However, it has been shown that recruitment of lymphoid precursor cells (LPCs) from the bloodstream into the thymus is not impaired during aging and that the expression of P-selectin and CCL25 is not reduced [23]. Also, LPCs from aging mice could normally seed the young thymus in a system of kidney capsule transplantation [23].

Besides ETPs, many studies have highlighted further age-related changes within the later stages of DN thymocyte development, with the observation of a decrease in the proportion of DN2 (CD44⁺CD25) and DN3 (CD44⁺CD25⁺) cells [24]. DN fluctuation seems to directly impact the generation of double-positive thymocytes, the subsequent stage of thymocyte development. A worsening communication between the cortical and medullary thymic microenvironments with developing thymocytes during aging also results in the loss of self-versus non-self-recognition, due to failure of selection processes, and decreased or an inherent inability for T-cell receptor rearrangement [24].

With respect to mature thymocytes, although the thymic export of both CD4 and CD8 T cells has been reported to be altered during senescence, observations in humans and murine models have outlined a more dramatic decline in the CD8 subset [25], suggesting that aging-induced alterations in the thymic microenvironment differentially regulate the development of conventional T cells.

Role of TEC-Mediated Cytokine and Hormonal Signaling during Thymic Senescence

Many cytokines and hormones are secreted by the thymic microenvironment, providing constitutive processes such as migration and development of thymocytes, in addition to regulating cell numbers in the T-cell pools [26–28]. Age-dependent TEC transformations result in an altered secretory pattern that seems to underlie different aspects of thymic involution, as will be discussed below.

IL-6 Family Cytokines

Cytokines of the IL-6 proinflammatory family produced by TEC include IL-6, leukemia inhibitory factor and oncostatin M. Studies evaluating and profiling cytokines in human thymus during aging have demonstrated a shift in cytokine expression patterns to predominantly IL-6 family cytokines by thymic adipocytes and TECs [29]. The increased expression of leukemia inhibitory factor and IL-6 further correlates with decreased production of recent thymic emigrants. Importantly, the administration of leukemia inhibitory factor, oncostatin M or IL-6 in mice results in thymic involution [29], suggesting a putative role of inflammation in thymus atrophy during aging.

Transforming Growth Factor β

Parallel to the cytokines of the IL-6 family, the intrathymic expression of transforming growth factor β (TGF- β) is also increased during aging [29]. TGF- β signaling plays an age-dependent negative role in controlling thymic aging and cellularity, inhibiting the IL-1-, IL-2- and IL-7-dependent thymocyte proliferation [30] as well as the maturation of the double-positive subset [31]. The physiological process of thymic senescence is attenuated in mice deficient for the expression of TGF- β RII on TECs but not on thymocytes [32]. The lack of TGF- β -mediated signaling in TECs is correlated with an increase in the absolute number of thymocytes during puberty, and it also improves thymic reconstitution after irradiation [32]. These data reveal that, rather than directly affecting thymocytes, TGF- β acts on the thymic microenvironment, modulating TEC capacity to support thymopoiesis. On the other hand, it has been shown that a block in canonical TGF- β signaling by the loss of Smad4 expression in TECs leads to qualitative changes in TEC function and a progressively disorganized thymic microenvironment [33]. Although TGF- β signaling exerts a main role in the induction of fibrosis in many organs [34], it is unclear if the fibrotic and fatty changes that culminate in TEC transformation into adipose tissue during aging are related to the local increase of this cytokine.

Interleukin 7

IL-7 signaling is required to induce T-cell receptor β rearrangement in DN thymocytes and for the survival of these cells [35]. Accordingly, IL-7-deficient mice present a blockage at the DN1 to DN2 transition and subsequent loss of thymic output [36]. Since these changes are also observed in old mice, it is hypothesized that decreased IL-7 signaling in the thymus is involved in age-related

thymic involution. In this respect, the intrathymic expression of IL-7 declines with aging in the thymic microenvironment [37], whereas neutralization of IL-7 in young mice induces reversible thymus atrophy [38]. The specific role of locally produced IL-7 in thymic involution during aging was assessed with the injection of IL-7-secreting stromal cells into the thymi of recipient aging mice [39]. Although the increased local concentration of IL-7 maintained the first step of thymopoiesis at a level far higher than was seen in age-matched controls, there was no decrease in thymic involution or increase in T-cell output. Together, these data indicate that at least during aging, systemic IL-7 seems to influence thymic involution more directly than within the local thymic circuitry.

GH and IGF-1

We have performed a series of studies regarding the direct and indirect effects of GH on thymocytes and thymic microenvironment [for a review, see 40]. GH enhances thymic function through the activation of its receptors in TEC and thymocytes, a mechanism mediated by IGF-1. Exogenous GH stimulates proliferation of both cell types and an enhancement in the secretion of thymic hormones, cytokines, and chemokines by the thymic microenvironment. This leads to increased T-cell intrathymic trafficking and export, possibly by increasing the production of extracellular matrix and chemokines [41].

In addition to the endocrine role of GH in the thymus, the organ itself is able to produce and secrete GH by both developing thymocytes and TEC [40]. In fact, thymic GH contents diminish with advancing age, similar to systemic GH levels [42]. Accordingly, it is possible that thymus-derived GH is also required for thymic maintenance.

Luteinizing Hormone-Releasing Hormone

We have previously demonstrated the use of luteinizing hormone-releasing hormone agonists (LHRH-A) for the enhancement of T-cell recovery following autologous and allogeneic BM transplantation [6, 43]. The chronic administration of LHRH-A downregulates the expression of its own receptor by the pituitary, which in turn downregulates the production of LH and FSH, leading to a blockade of sex hormone release. This has been demonstrated to result in beneficial effects on the BM and thymus, with increased thymopoiesis and naive T-cell production [6]. The role of locally produced LHRH in thymus senescence is not yet elucidated; however, intrathymic production of LHRH increases in castrated mice, whereas testosterone replacement prevents this effect [44].

Fig. 2. Possible mechanisms by which therapies based on the stimulation of FoxN1 and activation of thymic epithelial precursor cells would be able to induce a sustained recovery of the aging thymus. **a** The therapies currently applied in clinics to enhance T-cell reconstitution are based on the exogenous administration of LHRH, IL-7, or GH, but they result in a transient rejuvenation of thymopoiesis. **b** Therapies manipulating the expression of FoxN1 in the aging thymus induce improved TEC recovery and thymopoiesis. FoxN1 overexpression induces TEC proliferation (mainly TEC^{hi}) and inhibits EMT, which results in adipocyte (A) formation from fibroblasts (F) converted from TECs (T). It has been suggested that FoxN1 has a central role in the induction of functionality and survival of a putative TEPC population. **c** A third possibility for cell-based therapy derives from the *in vitro* generation of TEPCs from hESCs or by reprogramming fibroblasts by forced FoxN1 expression. Further, the recent characterization of TEPCs in the adult thymus [47] with self-renewal and colony-forming potential, characterized as TEC^{lo}, demonstrated that once activated, this subset could generate cTEC^{hi} and mTEC^{lo}, and indirectly mTEC^{hi} from mTEC^{lo}. This potentially enables a novel strategy for therapeutic thymic regeneration by targeting endogenous TEPCs.

Approaches for Thymic Regeneration

The therapies currently applied in preclinical and clinical studies to enhance T-cell reconstitution are based on the exogenous administration of LHRH-A, IL-7, IL-22, KGF and GH [for a review, see [45]. However, these treatments rely on substantial preexisting thymic tissue to support increased thymopoiesis, and the nature of TEC regeneration with LHRH-A may be transient (fig. 2a) [46]. In situations of significant thymic tissue damage, as

may be the case with graft versus host disease or following multiple cytoablative treatments, it may be necessary to develop new approaches that focus on TEC regeneration for sustained thymic recovery (fig. 2). In this way, strategies to manipulate the expression of the critical TEC transcription factor forkhead box n1 (FoxN1) or the activation of TEPCs, recently identified in the adult thymus [47], provide novel strategies to potentially generate more sustained thymus rejuvenation in aging individuals. In addition, protocols have been developed for the directed

Table 2. Efforts to rejuvenate the thymus through the manipulation of FoxN1 and generation of TEPCs from pluripotent stem cells

Authors	Methods	Results
<i>FoxN1 increase</i> Zook et al. [56]	Foxn1 transgenic mice (Foxn1Tg)	Attenuation of age-associated thymic involution Prevention of the expansion of peripheral CD4 memory T cells Attenuation in the decline of CD4 ⁺ and CD8 ⁺ naive cells
Garfin et al. [55]	Inactivation of Rb family genes in young mice	Increased FoxN1 expression Prevention of thymic involution during aging Increased production of naive T cells
Bredenkamp et al. [57]	TEC-specific increase of FoxN1 in the thymus of aging mice	Increased thymopoiesis Increased naive T-cell output Increasing proliferation of progenitor TEC (MHCII ^{lo})
<i>TEPC generation</i> Lai and Jin [51]	Generation of TEPC from mESC	Reconstitution of the normal thymic architecture in vivo Thymocyte regeneration after BM transplantation Increased number of functional naive splenic T cells
Lai et al. [70]	mESC-derived TEPC transplantation in mice after allogeneic BM transplantation	Efficient establishment of thymocyte chimerism Generation of naive T cells in both young and old recipients No induction of GVHD and GVT activity enhanced
Parent et al. [49]	Generation of TEPC from hESC	T cell development upon transplantation into nude mice Developed T cells capable of in vitro proliferation as well as in vivo immune responses
Sun et al. [50]	hESC-derived TEPC transplantation in NOD/SCID mice engrafted with HSCs	In vivo differentiation into TEC expressing MHCII, AIRE, and FoxN1 Human T cell generation in NOD/SCID mice engrafted with human HSCs

GVHD = Graft-versus-host disease; GVT = graft versus tumor; AIRE = autoimmune regulator; NOD/SCID = nonobese diabetic/severe combined immunodeficiency.

differentiation of pluripotent stem cells into TEPC in mice and humans [48–51], for the potential transplantation of de novo generated TEPC. These findings are summarized in table 2 and reviewed below.

Manipulation of FoxN1 Expression

FoxN1 is a transcription factor expressed in the thymus and skin epithelium. In the thymus, it is expressed exclusively by TECs and essential for thymic development [52]. Mutations in the FoxN1 gene in both humans and mice result in an athymic condition leading to severe immune deficiency. In affected patients with FoxN1 deficiency, the thymic lobes are still present, but thymopoiesis is completely blocked, leading to severe primary T-cell immunodeficiency and often death in early childhood due to severe infections [53]. Studies using a conditionally reversible null allele of *Foxn1* revealed that the expression of FoxN1 is required to differentiate the whole TEC network from TEPC and for the successful

colonization of thymic rudiment by BM-derived precursors [54].

In addition to its role in organogenesis, FoxN1 coordinates TEC proliferation in both embryo and adult thymus through the regulation of S-phase genes. In mice mutant for the tumor suppressor retinoblastoma protein, the increased activity of E2F transcription factors resulted in the increased expression of FoxN1 and consequent proliferation of TEC subsets, while the downmodulation of FoxN1 in this mutant inhibited thymic expansion (fig. 2b) [55]. Accordingly, FoxN1 overexpression in aging mice induces a higher proliferation of MHCII^{hi} TECs and prevents structural alterations associated with thymic involution [56]. Recently, Bredenkamp et al. [57] demonstrated that the single regeneration of FoxN1 during aging results in robust thymic recovery, characterized by an attenuated decline in early thymic progenitors and thymocyte numbers and paralleled by a higher number of recent thymic emigrants in the periphery.

FoxN1 impairment in thymic aging also seems to be related to the induction of TEC EMT and proadipose differentiation (fig. 2b). Accordingly, a prolonged decrease in FoxN1 and its regulator Wnt-4 leads to the degeneration of the thymic epithelial network [58], whereas Wnt-4 overexpression protects TECs against dexamethasone-induced senescence [59].

Consistent with the central role of FoxN1 in TEC homeostasis in the adult thymus through the induction of functionality and survival of TEPCs [60–62], Bredenkamp et al. [63] recently demonstrated that enforced FoxN1 expression alone was sufficient to reprogram mouse embryonic fibroblasts into functional TECs (iTECs). These cells were able to convert ETPs down the thymocyte lineage with CD4⁺CD8⁺ double-positive cells and CD4⁺ and CD8⁺ single-positive cells when cocultured in vitro. When reaggregated with embryonic thymic mesenchyme and immature mouse thymocytes and grafted under the kidney capsule of nude mice, the iTECs were able to differentiate into all TEC subsets including Aire⁺ cells and form correctly compartmentalized cortical and medullary regions. If this approach could directly translate to human cells using accessible patient tissue, it would potentially provide an autologous source of TECs for patients to be used either for direct transplantation or for in vitro generated autologous T cells (fig. 2c).

Activation of TEPC for Thymic Regeneration

The phenotypic identification and characterization of endogenous TEPCs in the thymus of adult mice has only recently been achieved [47], allowing a new field of exploration for the development of therapies targeting thymus rejuvenation.

Stem and progenitor cell populations are characterized by self-renewal and the capacity to give rise to different cell lineages. In epithelial tissue, homeostasis is maintained by epithelial stem cells via asymmetric division, enabling the repair of damaged tissues and self-renewal [64]. This process enables the progenitor cells to generate two different kinds of daughter cells: one continues to maintain multipotent potential, whereas the other differentiates into specialized cells responsible for the function of the tissue or organ.

TEC stem/progenitor cells have been identified in the early embryonic thymus (E12.5–E14.5) and are able to differentiate into the full range of thymic epithelial subsets, including Aire expressing mTEC [65–67]. Whether a common bipotent stem cell population exists in the postnatal thymus, which can form both cortical and medullary compartments to maintain homeostasis, has been

a controversial topic for some time; however, an emerging body of data supports their existence.

Recently, an adult thymic epithelial progenitor population within the TEC^{lo} subset has been identified and characterized as bearing the phenotype EpCAM⁺UEA-1⁻Ly51^{lo}MHCII^{lo}Sca1^{hi}α-6integrin^{hi} [47]. By combining cellular and molecular characteristics, in vivo turnover and functional analysis by 3D in vivo and in vitro culture systems, their self-renewal was demonstrated as well as their colony forming potential and generation of mature cortical and medullary cell lineages, including the Aire⁺ medullary subset. Whether FoxN1 expression is absolutely required in adult thymic epithelial stem cells has recently been challenged in an article by Ucar et al. [68]; however, this will no doubt continue to be debated.

By means of a transgenic label-retaining cell assay, another recent study revealed in neonatal and adult mice the existence of non-senescent, quiescent cTECs with putative features of progenitor cells, expressing relatively low levels of p16INK4a, p19ARF, and Serpine1 and high levels of Bmi1, Foxn1, Trp63, and Wnt4 [69]. These cells were only detectable after 16 weeks in adults, but not in neonate or young mice. Interestingly, it was demonstrated that the dramatic increase of the mTEC/cTEC ratio during the first weeks of life occurs independent of apoptosis or proliferation modulation, favoring the hypothesis of generation of mTEC phenotype from cTEC [69].

In summary, the identification of adult TEPC opens a new field to progress research into the molecular regulation of these cells during homeostasis, differentiation and age-dependent degeneration, besides their manipulation for regeneration following damage.

TEPC Generation from Pluripotent Stem Cells

Pluripotent stem cells hold great promise in the field of regenerative medicine. The ability to selectively induce the generation of TEPCs in vitro from ESCs or induced pluripotent stem cells for transplantation has important implications regarding the prevention and/or treatment of primary and secondary T-cell immunodeficiencies [50, 70].

The generation of TEPC from pluripotent stem cells was first demonstrated using mouse ESCs (mESCs), which were selectively induced in vitro to differentiate into cells that have the phenotype of TEPCs [51]. In this elegant study, Lai and Jin [50] demonstrated that, when placed in vivo, these mESC-derived TEPCs self-renew, develop into TECs, and reconstitute the normal thymic architecture. Functionally, these mESC-derived TEPCs enhanced thymocyte regeneration after BM transplanta-

tion and increased the number of functional naive splenic T cells. Later, Lai et al. [70] showed that transplantation of mESC-derived TEPCs resulted in the efficient generation of naive T cells in both young and old recipients following allogeneic BM transplantation.

The development of protocols for human ESCs (hESCs) to generate TECs capable of supporting T-cell development, followed a more complex process of mimicking thymus organogenesis [49, 50]. Sun et al. [50] directed the differentiation of hESCs into TEPCs by sequential regulation of Activin, retinoic acid, BMP, and WNT signals to initially differentiate cells into definitive endoderm and then generate TEPC identified by the expression of FoxN1 [49]. Cells underwent final development in vivo, with the induced thymic epithelium expressing the functional thymic markers MHCII and AIRE. These human TECs could support mouse thymopoiesis in T-cell-deficient mice and promote human T-cell generation in nonobese diabetic/severe combined immunodeficiency mice. The study by Parent et al. [50] included a more precise regulation of TGF- β , BMP4, retinoic acid, Wnt, Shh, and FGF, signaling the maturation of hESC-derived TEPC into functional TECs that supported T-cell development [49]. Importantly, the engrafted TEPCs produced T cells capable of in vitro proliferation as well as in vivo immune responses. A third study using a FoxN1-GFP/w hESC reporter line cultured hESC in high levels of Activin A to form definitive endoderm followed by KGF under serum-free conditions [48]. The generation of FoxN1⁺ (GFP⁺) thymic endodermal progenitors was achieved through the sequential expression of genes involved in endoderm and thymus development. Through the transcriptional profiling of purified FoxN1-GFP⁺ cells, Soh et al. [48] identified several combinations of cell-surface markers as ITGB4, HLA-DR, and EpCAM that could selectively isolate FoxN1⁺ TEC progenitor populations derived from unmodified ESC lines. This protocol was also demonstrated to be reproducible using alternative hESC lines and an induced pluripotent stem cell line. Thus, pluripotent stem cell-derived TEPC grafts, using the patient's own cells, may lead to broad applications for restoring immunity following irreversible thymic damage, or age-related thymic decline (fig. 2c).

Conclusion

The rejuvenation of the immune system can be achieved by therapies focused on thymus reestablishment. Like in other epithelial tissues, the postnatal thy-

mus appears to preserve an intrinsic ability to regenerate by responding to external stimuli, such as sex steroid ablation and cytokine administration. In particular, the recovery of the TEC compartment is a determinant for sustained restoration of thymopoiesis. Current approaches focus on the modulation of growth factors and hormones secreted by TECs, and therefore only promote a partial and transient thymus recovery. Thus, the emergence of new strategies based on TEC recovery, such as inducing FoxN1 expression, TEPC activation and de novo generation of TEPC, have emerged as a new generation of strategic interventions focused on TEC reestablishment.

The recent identification of progenitor markers and their consecutive expression pattern in the adult thymus is an important step in the analysis of the prospective potential of TEPCs as well as for establishing strategies to regenerate a functional thymus with full cortical and medullary compartments. Further, the actual results achieved with the development of TEPCs from pluripotent stem cells able to generate functional human T cells in vivo has pioneered a new field of clinical and therapeutic possibilities for the treatment of pathologies related to T cell-associated immunosuppression. Regarding the potential manipulation of FoxN1 for TEC generation, the identification of the precise pathways that modulate the expression of this transcription factor provide a new paradigm for regenerative biology strategies. The combination of strategies focusing on both TEC and lymphoid regeneration may achieve a therapeutic threshold that will successfully enhance full immune recovery following cytoablative treatments in the aged and elderly.

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