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Thymus: The Next (Re)Generation

Mohammed S. Chaudhry[1], **Enrico Velardi**[1], **Jarrod A. Dudakov**[1,3], and **Marcel R.M. van den Brink**[1,2,4]

[1]Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065

[2]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065

[3]Program in Immunology, Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

[4]Department of Immunology and Microbial Pathogenesis, Weill Cornell Medical College, New York, NY 10021

Summary

As the primary site of T cell development, the thymus plays a key role in the generation of a strong yet self-tolerant adaptive immune response, essential in the face of the potential threat from pathogens or neoplasia. As the importance of the role of the thymus has grown, so too has the understanding that it is extremely sensitive to both acute and chronic injury. The thymus undergoes rapid degeneration following a range of toxic insults, and also involutes as part of the aging process, albeit at a faster rate than many other tissues. The thymus is, however, capable of regenerating, restoring its function to a degree. Potential mechanisms for this endogenous thymic regeneration include keratinocyte growth factor (KGF) signaling, and a more recently described pathway in which innate lymphoid cells produce interleukin-22 (IL-22) in response to loss of double positive thymocytes and upregulation of IL-23 by dendritic cells. Endogenous repair is unable to fully restore the thymus, particularly in the aged population, and this paves the way towards the need for exogenous strategies to help regenerate or even replace thymic function. Therapies currently in clinical trials include KGF, use of the cytokines IL-7 and IL-22, and hormonal modulation including growth hormone administration and sex steroid inhibition. Further novel strategies are emerging in the pre-clinical setting, including the use of precursor T cells and thymus bioengineering. The use of such strategies offers hope that for many patients, the next regeneration of their thymus is a step closer.

Keywords

Thymus damage; Aging; Tissue Regeneration

Address correspondence to vandenbm@mskcc.org. Box 111, 1275 York Ave, New York, NY 10065. P: 646-888-2304, F: 646-422-0298.

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Introduction

The thymus is the primary site of T cell development. As other reviews in this volume have highlighted, the specialized thymic microenvironment supports the development of a broad but self-tolerant T cell repertoire. This is vital to the development of a strong adaptive immune response against pathogens and tumours, without leading to autoimmune disease.

The importance of the thymus, however, must be reconciled with the potential for loss of thymic function over a lifetime, and the ensuing detrimental effects. The thymus is exquisitely sensitive to a range of acute insults. It is important to stress that these insults should not be considered in isolation, as significant potential exists for coincidental conditions to impair thymic function in the clinical setting. Hematopoietic stem cell transplantation (HSCT), for example, may acutely damage the thymus through the chemotherapy, radiotherapy and antibody therapy of the conditioning regime. This may be compounded by infections acquired by the immunosuppressed patient, and in the case of allogeneic HSCT, thymic graft versus host disease (GVHD). Following resolution of the acute insult, the thymus is, however, capable of intrinsic recovery.

In addition to acute degeneration, thymic decline also occurs as an inevitable chronic process, in which the thymus gland undergoes involution with age. Thymic involution differs from aging in other organs and cannot be reversed. Furthermore, the aging process impairs the ability of the thymus to regenerate from acute damage. There is thus an increasing recognized need for exogenous strategies that can rejuvenate the aged or damaged thymus. We review the most promising therapeutic avenues, some of which are now entering clinical trials. There are caveats to such approaches, however. There may be potential detrimental consequences to rejuvenating an organ that has been evolutionarily selected to involute with age. Nevertheless, thymic regeneration undoubtedly offers much therapeutic potential, and the ability to harness this epitomises the exciting intersection between regenerative medicine and immune biology.

Causes and targets of acute thymic damage

Notwithstanding its importance for generating a diverse T cell repertoire, the thymus is extremely sensitive to negative stimuli (Figure 1). However, despite this sensitivity, thymic regeneration can occur following resolution of the insult; although this ability is blunted with increasing age (1). Acute thymic damage can cause significant morbidity and mortality in conditions where active recovery of thymopoiesis is required to sustain immune competence, such as after clinically induced immune depletion (2), and has been directly linked to opportunistic infections and an adverse clinical outcome in recipients of allogeneic HSCT (3).

Cytoablative therapies

Although primarily directed against malignant cells, chemotherapy can target the haematopoietic system, including the T cell compartment (4, 5). Such T cell immunodeficiency results from ablation of both thymic and peripheral T cell subsets. Within the thymus, alkylating agents such as cyclophosphamide have been shown to deplete all

thymocyte subsets (6, 7). While thymic stromal cells were traditionally thought to be resistant to damage caused by chemotherapy or radiation, there is now considerable evidence to suggest that such agents can also damage the thymic stroma (8). Thymic epithelial cells (TECs) expressing the highest levels of MHC class II are particularly vulnerable to the effects of chemotherapy, particularly those located in the medulla (mTECs) (8), likely due to their higher rate of proliferation (9, 10). Since these cells play a key role in negative selection (11), this selective depletion may have profound implications for causing a lack of tolerance to self, following such treatments, although this remains hypothetical at present.

Following chemotherapy, homeostatic expansion of the peripheral T cell pool can facilitate $CD8^+$ T cell recovery within months in both young and old patients (12), although clearly the repertoire diversity is impacted (13). However, while in younger patients, peripheral expansion is augmented by recovery of thymic function in older patients there is evidence that chemotherapy induced thymic damage may take years, if ever, to recover (5, 14, 15).

Similarly, radiation can induce acute thymic damage and loss of cellularity (16). Although all thymocyte populations are typically affected, the double positive (DP) thymocyte population is particularly sensitive to irradiation (17). However, like chemotherapy, radiation can also lead to stromal damage with considerable reduction in TEC numbers (16, 18). It is notable that certain cell populations, such as endothelial cells and innate lymphoid cells (ILCs), are relatively radio-resistant (19, 20), and there cells can play a role in endogenous thymic regeneration.

Steroid hormones

Glucocorticoid hormones, acting through the nuclear glucocorticoid receptor (GR), exert a wide range of immunosuppressive and anti-inflammatory effects, providing the rationale for their use in autoimmune disease, allergic and inflammatory disorders, allograft rejection, GVHD and lymphoid malignancies (21). Similarly, elevated levels of endogenous glucocorticoids, as produced in stress, starvation, infection and Cushing disease, may also lead to immunosuppression (22). In the thymus, glucocorticoids induce apoptosis of DP thymocytes, which preferentially express the GR (23), in an Apaf-1 and caspase-9 dependent manner, leading to acute involution (24, 25). In view of the wide range of conditions associated with their elevated levels, glucocorticoids thus represent a common pathway mediating many episodes of acute atrophy of the thymus. Interestingly, the thymic epithelium itself can also produce glucocorticoids (26), and this may have subtle effects on steady-state thymopoiesis. For instance, thymus-derived glucocorticoids can inhibit T cell receptor (TCR)-mediated deletion of DP thymocytes, and thus modulate which TCR avidities result in positive or negative selection (27, 28).

Increased levels of the sex steroid hormones, testosterone, progesterone and estrogen, likewise act through their nuclear receptors causing thymic involution. Their effect is most obviously manifest during puberty, during which the rate of thymic involution increases rapidly (29). Similarly, pregnancy is associated with an acute transient involution of the thymus (30). Sex steroids have multiple effects to reduce the lymphocyte pool within the thymus. Although sex steroids can directly induce apoptosis of thymocytes (31), studies using chimeras and androgen-resistant testicular feminization mice have demonstrated that

the primary mediator of sex steroid-induced thymic involution are the non-hematopoietic stromal cells (32). TECs express a functional androgen receptor (AR), and AR knock-down in this population partially abrogates sex steroid-induced thymic involution, further implicating TECs as important targets of androgens (33, 34). More recently it has been shown that androgens directly inhibit thymopoietic factors in TECs, including CCL25, a ligand critical for entry of T cell progenitors into the thymus, and Delta-like ligand 4 (Dll4), a Notch ligand crucial for the commitment and differentiation of T cell progenitors in a dose-dependent manner (35, 36). Furthermore, through their effects on both HSCs and the bone marrow microenvironment, sex steroids decrease lymphoid differentiation, reducing the number of available T cell progenitors in the thymus (37, 38).

Infection

Although previously thought to be an immune-privileged site (39), protected from infections and immune responses, increasing evidence over the last decade has highlighted that the thymus is indeed a target for a range of pathogens, including bacteria, viruses, fungi and parasites (40). The resulting lymphostromal disruption causes acute thymic involution and defects in thymic export of newly generated naïve T cells, leading to impaired immune responses against the pathogen (41). Thymic damage as a result of acute infection may be caused directly by the pathogen within the thymus or by the systemic effects invoked by infection.

Systemic pathogen specific factors most notably include bacterial lipopolysaccharide (LPS) released from gram-negative bacteria such as Escherichia coli (42, 43). LPS leads to severe acute thymic atrophy that peaks within 3–5 days, characterized by loss of DP thymocytes (44). In addition to the systemic effects of LPS, many infections also trigger a stress response and a surge in glucocorticoid levels, inducing thymocyte apoptosis as described above (25, 45). Other inflammatory mediators may rise during infection, leading to thymic damage, and again in particular depletion of the DP population. These include tumour necrosis factor α, the release of which can be triggered by infection with Francisella tularenis (46) and Trypanosoma cruzi infection (47); as well as the release of interferon γ caused by Salmonella enterica infection (48).

Pathogens may directly invade the thymus. HIV, the most well studied example, directly infects $CD4^+$ SP thymocytes and progenitors, and induces apoptosis of uninfected thymocytes through secretion of viral products (49, 50). In addition, HIV infects thymic stromal cells, including TECs and dendritic cells (51–53). This leads to disruption of thymic architecture and degradation of the thymic microenvironment. Similar effects on TECs are observed in other chronic viral infections, such as CMV (54). Direct infection of the thymus by Trypanosoma cruzi can also alter the thymic environment by increasing deposition of fibronectin and laminin and increasing the expression of the chemokine ligands CXCL12 and CCL4 by stromal cells. In addition to causing structural disruption to the thymus, these changes promote the migration of DP thymocytes, leading to their premature release from the thymus and further thymic involution (55).

Although thymic atrophy in the context of infection may be coincidental, the conservation of this phenomenon across a diverse range of pathogens suggests a greater significance. Thus,

induction of thymic involution may be a deliberate virulence strategy employed by pathogens in order to subvert immune responses, and propagate survival within the host. However, interestingly there is also emerging evidence in the context of mycobacterial infection that thymic infection in the absence of atrophy can result in the generation of naïve T cells tolerant to the pathogen (56). Thus, although it compromises T cell immunity, thymic atrophy may in fact represent a deliberate strategy employed by the host, to avoid the generation of an immune repertoire tolerant to certain pathogens. A further understanding of this process is therefore essential in the context of potential strategies to rejuvenate the damaged thymus.

Graft versus host disease

GVHD is a complication of allogeneic HSCT, characterised by three distinct phases: (1) tissue damage from conditioning therapy or other causes, (2) activation of alloreactive donor T cells by host antigen-presenting cells, and (3) target tissue damage mediated by soluble and cellular effectors (57, 58).

Although acute GVHD has been traditionally viewed as a disease of gut, liver and skin, there is now a large body of evidence demonstrating that the thymus is an extremely sensitive target of alloreactive T cells (59–62). Clinical manifestations of GVHD are associated with changes to both the lymphocyte and stromal compartments of the thymus, leading to acute involution. These include loss of cortical and medullary thymocytes, loss of TECs, decreased demarcation of the cortico-medullary junction and disrupted architecture of the gland (63, 64). Such changes have been shown to lead to decreased thymic output of naïve T cells, as indicated by TCR excision circles (TRECs), and a distorted TCR repertoire (65).

More recently, murine models of GVHD have demonstrated that loss of thymic cellularity is primary due to loss of the large DP subset, as a consequence of both a block in differentiation block in early DN thymocytes and also increased apoptotic cell death in the DP population (59). TECs have likewise been identified in these models as direct targets for alloreactive T cells, but in addition to acting as targets, there is also evidence that given their expression of MHCI and MHCII, these TECs can act as antigen presenting cells sufficient in themselves to prime alloreactive T cells (60). Together with the fact that the thymus is extremely sensitive to GVHD-mediated damage (62), this raises the distinct possibility that GVHD can be restricted to the thymus even during subclinical GVHD, and thus in many cases may not be detected in the clinical setting yet still have detrimental consequences for T cell reconstitution.

Murine models of GVHD have also highlighted that thymic damage in such cases is associated with impaired negative selection of thymocytes and Treg development, which may be potential contributory factors to the development of chronic GVHD and autoimmunity post HSCT (66, 67).

Moreover, given that corticosteroids are often used as a first line treatment for GVHD (58), and that they induce thymic involution in and of themselves, their use will likely compound the detrimental effects on thymic function during GVHD. Thus, the treatment of thymic GVHD is a conundrum that may require alternate therapeutic approaches.

Endogenous thymic regeneration

Endogenous thymic regeneration is a crucial function that allows for renewal of immune competence following immunodepletion caused by cytoreductive chemotherapy or radiation, acute infection and stress (Figure 1). Although the potential for the thymus to regenerate itself has been known for some time, surprisingly little is known about the mechanisms involved with this regeneration.

One of the first studies to identify a potential pathway of endogenous regeneration focused on fibroblast growth factor 7 (FGF7, also known as KGF). In this study, it was found that although KGF was redundant for steady-state thymopoiesis, it was crucial for regeneration after injury such as that caused by TBI, as mice deficient for KGF exhibited significantly worse thymic recovery compared to wildtype controls (68). KGF targets thymic epithelial cells and promotes their proliferation and differentiation (69, 70). Furthermore, KGF has been used in several studies and clinical trials as a means of boosting thymus function after damage (see below).

Another pathway of endogenous thymic regeneration identified is centered on ILCs and their production of interleukin-22 (IL-22), a recently identified cytokine predominantly associated with maintenance of barrier function at mucosal surfaces. This study proposed that 1) the depletion of $CD4^+CD8^+$ DP thymocytes triggers, 2) upregulation of IL-23 by dendritic cells (DCs), which induces 3) the production of IL-22 by intrathymic ILCs. IL-22 directly promotes the proliferation and survival of TECs, therefore this cascade of molecular and cellular events leads to regeneration of the supporting microenvironment and, ultimately, to rejuvenation of thymopoiesis (19). Additionally, a subsequent study correlated the increased expression of IL-22 after damage with increased expression of Foxn1 (71). Foxn1 is a molecule critically involved with thymus ontogeny and is also clearly important for thymic maintenance and regeneration (72, 73). In fact, forced expression of Foxn1 leads to regeneration of the aged thymus (74, 75) and thus activation of Foxn1 after damage could represent a potent pathway of endogenous or exogenous thymic regeneration. Although it is tempting to think that IL-22 may be directly promoting expression of Foxn1, currently there is no clear evidence of such a link.

It is also very likely that molecules involved in steady state thymopoiesis also contribute towards thymic regeneration. These include IL-7, CXCL12, SCF, CCL25, and the notch ligand DLL4. In fact, many of these have been identified as part of the mechanistic pathways for exogenous thymic regeneration through such systemic means as sex steroid ablation, which crucially needs IL-7 (but not KGF) to mediate regeneration (76), and induces expression of the Notch ligand Dll4 (36), CCL25, which induces importation of progenitors (35), and VEGF, which promotes vascularization and endothelial cell function and is also likely involved in endogenous thymic regeneration (77, 78).

Chronic thymic damage: Involution of the thymus with age

Aging is an inevitable process occurring in all living organisms, associated with the progressive decline of function in tissues and increased susceptibility to disease (79).

Specific to the immune system, advancing age is associated with an array of defects in both innate and adaptive immunity, collectively termed immunosenescence, which impairs the ability to respond to both pathogens and vaccinations (80, 81). Elderly subjects demonstrate increased incidence of a range of bacterial and viral infections, and exhibit increased mortality from these diseases in comparison to younger patients (82). Furthermore, immunosenescence may also lead to a loss of tumour immune surveillance, increasing the propensity for neoplastic disease in the elderly (83). There may also be immune dysregulation, such that older subjects experience increasing autoimmune disease (84). A key feature of immunosenescence is involution of the thymus, characterized by both a progressive decrease in thymic cellularity and a loss of tissue organization (29, 85). This leads to profound age related defects, both quantitative and qualitative, in the T cell compartment.

Consequences of thymic immunosenescence

Involution of the thymus with age ultimately causes a decrease in the thymic output of naïve T cells and subsequently a constriction of the peripheral TCR repertoire. As T cell production decreases, there is homeostatic expansion of existing peripheral T cells, which leads to a skewing towards an increased proportion of memory T cells, and a consequent reduction in the diversity of TCR repertoire (86, 87). However, even those naïve T cells that are produced in the aged thymus are functionally impaired; expressing higher levels of senescence markers such as CD57 and exhibiting limited proliferation in response to antigen stimulation (88). Furthermore, they have reduced homing receptors such as CD62L and CCR7, interfering with their mobilization to relevant sites of action (89). The overall consequence of these changes is a defective adaptive immune response to neo-antigens, including new pathogens, vaccinations and tumour-associated antigens (90).

Thymic involution also contributes to the development of autoimmune disease in older age. The thymus provides a site where naïve T cells reactive to self are deleted through negative selection, both by interaction with thymic epithelial cells (TECs) and thymic dendritic cells. As these thymic cells are lost with age, the ability to mediate this central tolerance is impaired, and there is a greater chance that auto-reactive T cells will be released into the periphery (91, 92).

It is important to note, however, that despite thymic involution and alterations to the T cell compartment, some residual thymic function does persist into old age. Significant levels of naïve T cells can be detected in centenarians (93) and high levels of TRECs can still be detected in elderly subjects (94). Similarly, TDT expression within the thymus can be shown in aging persons (95). This residual function highlights that the thymus gland does not become a vestigial remnant in later life, but instead continues to function, albeit at a lower rate. Nevertheless, this low level function may be readily lost in the face of the acute insults described above, many of which are more common in the elderly population. This is compounded by a reduced ability of the aged thymus to endogenously regenerate following acute damage, although the reasons for this are not clear. The need to develop strategies to rejuvenate or replace thymic function, as discussed below, is thus particularly relevant to the aging population.

Mechanisms of age related thymic involution

There are phenotypic changes within both lymphoid and stromal compartments during age-related thymic involution, at least some of which will be exacerbated due to the extensive support offered by crosstalk interactions (96). The most prominent change in the thymus with age is the significant progressive decrease in the number of thymocytes within the organ. It has been estimated that in mice, the thymus of a 24 month-old mouse contains <1% of the T cells found in a neonatal equivalent.

Thymopoiesis is characterized by the stepwise differentiation of pro-T cells, the most primitive which is the early T-lineage progenitor (ETPs), itself a direct descendent of the circulating BM-derived progenitor (97). With age, although the number of bone marrow hematopoietic stem cells (HSCs) increases, their function, particularly towards lymphoid differentiation, declines considerably (98, 99). Although the ability of aged progenitors to get in to the thymus, and the ability of the thymus to accept these circulating progenitors, remains unchanged with age (100), this decrease in the supply of BM-derived progenitors results in a significant reduction in the number of ETPs in older individuals (101).

In addition to these numerical deficiencies, functionally, ETPs exhibit reduced proliferation and differentiation potential in the aged thymus (101). Similarly, later stages of DN thymocyte development are also affected, including differentiation blocks at the DN2, DN3 and DN4 stages (102, 103) and the accumulation of an abnormal population of $CD44^{+}CD24^{-}CD3^{+}$ DN cells in the thymus of older mice (102, 104). Although the significance of these cells remains unclear, a similar population has been found in the aged bone marrow, associated with a reduction in haematopoiesis (105). Aged DP and single positive (SP) thymocytes express reduced levels of CD3, possibly impairing TCR-signalling, and exhibit a decrease in Concanavalin A-induced proliferation (104).

Such defects in thymocyte proliferation and differentiation at all stages may represent intrinsic abnormalities within lymphocytes derived from an aged bone marrow. However, thymocyte development is also instructed by the thymic microenvironment as part of the lymphocyte-stromal cross talk. Thus, defective thymocyte development is also due in part to abnormal extrinsic signalling from the altered thymic environment. Highlighting these extrinsic factors influencing thymic involution, young ETPs, administered by intrathymic injection, are capable of developing in young mice but fail to do so in older recipients (106). Architecturally, the non-hematopoietic stromal microenvironment of the thymus undergoes marked structural changes with age, primarily within the TEC compartment. With age, the cortical and medullary thymic epithelial regions become irregular and atrophied, with loss of the definition of the cortico-medullary junction (107); there is diminution of the thymic epithelial space and enlargement of the perivascular space (29); and increased adiposity (108, 109). Underlying some of these changes within the TEC compartment is the reduced expression of the transcription factor FOXN1, a key regulator of TEC differentiation in the fetal and postnatal thymus (72, 110). Indeed, induced expression of FOXN1 in murine models has been shown to delay age related thymic involution (75), and can also robustly rejuvenate the aged thymus through expansion of the TEC compartment (74). In addition, TECs exhibit decreased MHC class II expression, attenuating their ability to interact with developing thymocytes (9), and their production of the thymopoietic cytokine IL-7 by TECs

is also reduced with age (111). Similar to the aging of many other organs, there is also an accumulation of fibroblasts in the aged thymus, leading to progressive fibrosis with age and again impeding the normal architecture of the gland (107). The accumulation of abnormal mesenchymal cells may also lead to abnormal signalling, such as adipokine secretion by adipocytes, which may further interfere with thymopoiesis, and warrants further investigation (109). The overall consequence of these structural changes is a progressive loss of thymic niche spaces, and thus a reduction in thymocyte development.

Underlying causes of age related thymic involution

Although aging affects all aspects of an organism, the thymus exhibits a unique pattern of aging, distinct from other tissues and organs (112). Thymic involution occurs much earlier than other acknowledged features of aging, beginning in early childhood and peaking at puberty (29). Following this initial phase, thymic tissue is estimated to be lost at approximately 3% per year until middle age and then subsequently at 1% per year (113). There is, however, individual variation, and in addition a sexual dimorphism exists, such that the rate of involution is greater in males than in females (114). Thus, although a full discussion of the causes of aging in general are beyond the scope of this review, below we focus on those aetiological factors which are particularly relevant to this pattern of involution observed in the thymus.

The variation in thymic involution between different strains of mice highlights how genetic polymorphisms may influence this process. This area has been investigated further through the use of recombinant-inbred (RI) mice (115). C57BL/6 and DBA/2 mice were used to generate 18 strains of RI mice, termed BXD. Using mathematical modelling, these mice could be classified into four groups on the basis of their initial thymic mass and the subsequent rate of thymic involution. A higher rate of thymic involution was shown to be in part due to a block in thymocyte development and thus decreased thymopoiesis. Quantitative trait loci influencing the rate of thymic involution could be identified most strongly on chromosome 9. The genes in this region thus represent a future target for further investigation.

Hormones, produced through the hypothalamic-pituitary axis, can influence age related thymic involution. Several studies have shown that growth hormone (GH) can rejuvenate the aged thymus (see below). The corollary of this has been to invoke the decline in serum GH concentration with age as playing a role in thymic involution.

The thymus is known to be acutely sensitive to sex steroids (see above), and similar lines of correlative evidence have strongly linked changes in sex steroid levels, especially androgens, to thymic degeneration with age. Notably, the greatest rate of thymic involution with age is observed at puberty (29), and sex steroid ablation is found to rejuvenate the aged thymus (see below). Furthermore, the increased level of androgens in males offers an explanation for the increased rate of thymic involution evident in males (114).

However, caveats exist to ascribing age related thymic involution solely to the changes in hormones with age. As discussed, the initiation of thymic involution begins in childhood, prior to the fall in serum GH or puberty associated rise in sex steroids. Furthermore, thymic

involution continues with age even when sex steroid levels fall (116). Thus, further aetiological factors are required to explain the pattern of aging observed in the thymus.

The accumulated damage from oxygen free radicals is a recognised contributor to the aging process in many organ systems, leading to chronic damage evident in later life. Such free radical damage may play a key role in the aging of the thymus. This is supported through the finding that reducing metabolic activity, for example by caloric restriction (117) or modulation of IGF signalling (118), can reduce thymic involution. It is notable that the rapid course of involution demonstrated in the thymus would be difficult to reconcile with chronic free radical damage. However, it has recently been shown that thymic stromal cells are deficient in the enzyme catalase, making these cells much more sensitive to damage by oxidative by-products and thus offering an explanation as to why thymic involution proceeds more rapidly than aging in other organs (119).

Is there a physiological purpose of age related thymic involution?

The unique response of the thymus to aging, both in terms of age at initiation and rate of involution, raises the question that there may be a hitherto yet undiscovered biological purpose to thymic involution. This concept is supported by the finding that thymic involution with age is an evolutionary conserved process, evident across most species.

It has been shown that with age, there is decreased influx into the thymus of lymphoid progenitor cells from the bone marrow, and this is associated with a risk of developing T cell leukaemia, due to excessive prolongation of the time developing thymocytes reside in thymic niches (120). It is thus speculated that thymic involution is needed to prevent the development of a pro-leukemic environment within the thymus (121). Alternatively, thymic involution may represent a trade-off in terms of bioenergetics. In youth, it is important to develop broad TCR diversity for the purposes of a strong adaptive immune system. Once developed, however, an organism may need to give priorities to other functions, such as reproductive capability, and thus reducing the energy consumption associated with thymic function is a requisite to achieve this.

Thus, although rejuvenation of the aged thymus undoubtedly offers therapeutic potential, there are caveats to be considered. Novel strategies to enhance thymic function in the aged individual will need to be performed carefully, both at the appropriate time and in the correct context.

Exogenous strategies to enhance thymic recovery

Rejuvenation of immune function remains a prominent unmet need in several clinical situations. Over the past decades, the mechanisms that regulate thymic recovery after injuries and the development of strategies that can boost its endogenous repair have been the focus of intensive research. Here we summarize preclinical and clinical studies of promising strategies that have the potential to be translated into novel regenerative therapies.

Keratinocyte Growth Factor (KGF)

Keratinocyte Growth Factor (KGF), also know as fibroblast growth factor 7 (FGF-7), is a protein with a predicted molecular weight of 22.5kDa that belongs to the fibroblast growth factor family. It is primarily secreted in a paracrine fashion from mesenchymal cells and promotes proliferation of epithelial cells (122). KGF binds to an epithelial cell–specific splice variant of the fibroblast growth factor receptor-2 (FgfR2-IIIb) expressed on TECs within the thymus. The FgfR2-IIIb receptor is activated not only by KGF but also by other close members of the family (FGF-1, FGF-3, and FGF-10) (123, 124). Although mice that are deficient for KGF show no signs of defective steady-state thymopoiesis, KGF KO mice show a deficit in thymic recovery after immune insults, such as sub-lethal radiation and HSCT.

As a result of these studies exogenous administration of recombinant KGF has been tested for its ability to enhance thymic regeneration, particularly in the setting of HSCT. KGF protects epithelial cells from several thymic injuries, including radio- and chemotherapy, allogeneic or syngeneic HSCT and GVHD (125–127). The mechanism by which KGF acts appears to be in promoting TEC proliferation, as BrdU incorporation in TECs increases up to 10 fold when mice are treated with KGF for 3 days (128). Ultimately this increase in TEC proliferation leads to increased thymocyte expansion and enhanced T cell export. Mechanistically, engagement of the FgfR2-IIIb promotes stimulation of the p53 and NF-kB pathways, and results in the upregulation of BMP2, BMP4, Wnt5b, and Wnt10b in young mice (128). A recent study in a non-human primate model showed that adult rhesus macaques, receiving KGF after autologous HSCT, had accelerated hematopoietic recovery, improved thymopoiesis (as evaluated by TREC analysis) and enhanced naïve T cell recovery following transplant. However, the increase in T cell recovery was not associated with an improved immunity against CMV reactivation or an improved response to tetanus toxoid vaccination (129). However, while KGF can also promote the expansion of young and old thymi in mice (68), there is some debate as to its effectiveness in promoting steady-state thymopoiesis in non-human primates (129).

Human recombinant KGF (Palifermin) is an FDA approved drug for the prevention of mucositis in recipients of high dose chemotherapy. However there are as yet no clinical studies designed to evaluate the sole efficacy of KGF in enhancing thymus function and T cell reconstitution in immunocompromised patients (127).

Interleukin-7 (IL-7)

IL-7 is a common gamma-chain (γ-chain) cytokine of 25-kDa involved in several adaptive immune processes including thymopoiesis, B cell development, and lymph node organogenesis (130). It also represents a pro-survival factor for ILCs. In the thymus, IL-7 is primarily produced by cTECs, and to a lesser extent by fibroblasts. The IL-7 receptor (IL-7R) is a heterodimer complex consisting of the IL-7Rα (CD127) and the CD132 common γ-chain receptor. Although the γ-chain is expressed on all hematopoietic cells, IL-7Rα is almost exclusively expressed on lymphoid cells. In fact, IL-7 is a critical non-redundant cytokine for both T and B cell lymphopoiesis, and in the thymus IL-7R stimulation promotes proliferation, differentiation and survival of the developing

thymocytes. It has been suggested that the engagement of the IL-7R induces cell differentiation through Jak-Stat signalling, and cell proliferation and survival through the PIK3/Akt pathway (131). Consistent with this, a defect in the IL-7Rα chain or the common γ chain in humans results in the severe combined immunodeficiency syndrome (SCID) (132, 133).

Given its critical role in thymopoiesis and in promoting pro-survival signals to peripheral lymphocytes, IL-7 has been extensively studied for its potential to enhance recovery after immune-insults (130). Several studies have demonstrated the beneficial effects of exogenous administration of IL-7, which enhances thymopoiesis and export of recent thymic emigrants, in addition to increasing the homeostatic proliferation of mature peripheral T cells (134, 135). Such effects ultimately lead to accelerated T cell recovery after syngeneic and allogeneic HSCT (136–138). Furthermore, administration of IL-7 has also been reported to increase antigen-specific T cell responses to vaccination and viral infections (139, 140). A more recent report described the results of a phase 1 clinical trial of recombinant human hIL-7 (CYT107) in recipients of T cell depleted allogeneic HSCT (NCT00684008). This study shows that CD3, CD4 and CD8 counts are increased in hIL-7 treated patients, and while no significant effects were reported on thymic output as measured by analysis of recent thymic emigrants, patients receiving hIL-7 did show a broader TCR beta repertoire diversity compared to untreated patients (141).

Interleukin-22 (IL-22)

IL-22 is a cytokine that belongs to the IL-10 cytokine family. It has gained increasing interest recently for its potential tissue protective effect (142). In particular, IL-22 has been primarily implicated in promoting epithelial integrity and antimicrobial immunity at mucosal surfaces. IL-22 is mainly produced by Th17 cells and innate lymphoid cells (ILCs) and it binds to a heterodimeric cell surface receptor, IL-22R, composed of IL-10R2 and IL-22R1 subunits (143–148). IL-22R is expressed on cells of epithelial origin, such as keratinocytes, intestinal or lung epithelial cells, and hepatocytes, and it is absent on cells of the immune system (142, 149). The key role of IL-22 in mediating endogenous recovery of thymus function after acute damage is described in detail above, and this provides the rationale to use IL-22 treatment as a potential therapeutic option, to stimulate thymic recovery in immunocompromised patients (19).

Based on these findings, a phase IIa clinical study has recently been initiated to evaluate the safety and tolerability of human recombinant IL-22 (hrIL-22) in conjunction with systemic corticosteroids in the treatment of gastrointestinal acute GVHD in patients receiving HSCT (NCT02406651). Importantly, peripheral T cell counts will be evaluated as a part of the study, allowing for further investigation of hrIL-22 as an immune-boosting therapy.

Growth hormone (GH) and Insulin-like growth factor 1 (IGF-1)

Several neuroendocrine hormones have been shown to mediate important effects on the immune system. Growth hormone (GH), also known as somatotropin, is a peptide hormone primarily secreted by the anterior pituitary gland (150); although interestingly, a previous study has also reported that GH can be produced by ex-vivo isolated human thymocytes and

TECs (151). The potent effects of GH on thymic function have been extensively investigated using GH deficient mice, which exhibit defects in T cell development. However, given that the GH-receptor (GHR) is expressed by TECs as well as developing thymocytes (151), it is unclear which are the primary targets of these effects of GH. In addition, exogenous administration of recombinant GH has been found to promote thymus regrowth in several preclinical mouse models (152). GH administration promotes improved thymic cellularity, increased TCR diversity and enhanced recovery of the hematopoietic compartment in immunocompromised and aged animals (153, 154). Several intrinsic and extrinsic mechanisms have been identified for the beneficial effects of GH on thymic function such as enhanced proliferation of TECs and trafficking of common lymphoid progenitors (CLPs) into the thymus (155–157).

Mechanistically, it has been shown that GH activates the JAK2/Stat5 pathway leading to the expression of several downstream genes. Insulin-like growth factor 1 (IGF-1) has been described as the principal mediator of the biological effects of GH (158–160). IGF-1, sometimes referred as somatomedin C, is a protein with a structure similarity to insulin. IGF-1 is expressed by thymic stromal compartment, in particular by TECs and fibroblasts, while the IGF-1 receptor is expressed not only by the stromal component (mainly by the epithelial cells and fibroblasts) but also by the hematopoietic compartment (161).

Encouraging results have been generated in several clinical studies. Recombinant human GH enhances thymic recovery and immune reconstitution in HIV-infected patients (162). More recently, a phase 1 clinical trial was performed to test the safety and efficacy of GH in improving immune reconstitution post unrelated cord blood transplant (NCT00737113).

Sex steroid inhibition (SSI)

Surgical or chemical sex steroid inhibition (SSI) is a well-described approach to promote thymic growth in several pre-clinical and clinical studies (163, 164). In fact, several reports have shown that SSI promotes thymic enlargement in young as well as in old mice and promotes accelerated thymic recovery after immune insults, such as radio- and chemotherapy, syngeneic and allogeneic HSCT and GVHD (7, 76, 165, 166). Androgen deprivation has been the focus of extensive research for the treatment of prostate cancer patients, and in the past years several pharmacological treatments have been developed to block directly the effects of the androgen receptor on the target cells or to suppress the hypothalamus-pituitary-gonadal axis that systemically promotes the release of androgens from the gonads. Several of those therapeutic treatments have been investigated for their potential to reversibly inhibit sex steroids and promote thymic regrowth and immune reconstitution in mice and humans. Clinical studies using LHRH-agonists, which desensitize the LHRH-receptor and ultimately lead to the inhibition of luteinizing hormone (LH) and follicle stimulating hormone (FSH) release, showed that the treatment promoted accelerated engraftment post-HSCT and enhanced T cell reconstitution in autologous and allogeneic HSCT recipients (165). As a functional read out of the impact of the LHRH-agonist treatment on the thymic recovery, it was shown that the peripheral T cell repertoire was more diverse in patients receiving the agonist therapy (165).

Although the effects of SSI on thymic regrowth have been known for more than one century (167, 168), the exact mechanisms underlying these regenerative effects are still not completely understood, although recent studies have demonstrated that SSI can directly promote the expression of CCL25 (35) and the Notch ligand DLL4 (36), as well as promoting the function of hematopoietic stem and progenitor cells (37, 38, 169). Further work is needed to understand these mechanisms, as transient ablation of sex steroids clearly represents a feasible and appealing immune regenerative strategy. Currently, two clinical trials are on going to test the effects of the LHRH-Agonist (Lupron) alone or in combination with KGF (Palifermin) in promoting immune recovery of allo-HSCT patients (NCT01746849 and NCT01338987).

Precursor T cells

Generation of large numbers of precursor T (pre-T) cells, readily available to be infused at the time of HSCT, represents an interesting therapeutic approach to accelerate immune recovery in immunocompromised patients. The development of newly generated thymic-derived T cells can take several months after HSCT and several conditions, such as aging and GVHD, can delay this process even further (2). With the advent of the robust OP9-DL1 system for generating precursor T cells ex vivo (170), the concept of using these ex-vivo generated pre-T cells to expand and mature in the thymus thereby shortening the duration of immunodeficiency has been a promising one. Previous work has shown that pre-T cells can be generated using ex vivo co-culture of HSCs with ectopically transduced OP9-DLL1 or DLL4, two critical factors for thymocyte proliferation and commitment to T cell fate (171). Importantly, similar results were also obtained using a cell-free culture condition where recombinant DLL1 or DLL4 were immobilized on the culture dishes (172, 173).

The adoptive transfer of pre-T cells with T cell depleted BM or purified LSK into lethally irradiated recipients increases thymic cellularity, improves T cell chimerism and enhances peripheral T and NK reconstitution (174). While clearly pre-T cells help T cell reconstitution by providing a ready source of T cell progenitors, there is a also a recently reported secondary effect whereby pre-T cells, through the process of thymic crosstalk, can actually enhance thymic stromal function long after the ex vivo pre-T have transited through the thymus (175). Functionally, pre-T cell administration after HSCT can enhance resistance to L. monocytogenes and promote increased immune clearance to the A20 tumor cell line (174). Importantly, pre-T cells represent an “off the shelf” therapeutic strategy, which can be administered across MHC barriers, since the immature cells will be educated in the thymus of the recipients, and can also be used as vehicles for chimeric antigen receptors (176). In addition, pre-T cells can be also genetically engineered to recognize virus-specific as well as tumor-specific antigens for tumor immunotherapy.

Thymus bioengineering

In addition to the identification of strategies that can boost the recovery of residual thymic functionality, substantial progress has also been made over the past years to the engineering of a transplantable artificial thymus. In addition to representing a clinically relevant alternative for patients with minimal residual functionality (for example as a result of aging or repeated cycles of immunosuppressive treatments), thymus transplantation represents one

of the few therapeutic options for patients with particular congenital immune deficiencies that result in complete athymia (such as Digeorge syndrome) (177–179). There is thus a significant clinical need for the development of ex vivo generated thymic tissue, rather than solely relying on endogenous thymus rejuvenation.

Immense effort has been invested in the past decades in order to characterize and rebuild in vitro the complex 3D structure that confers the thymus its specialized microenvironment. A particularly important area of investigation is the identification of biomaterials that can reproduce the 3D artificial matrix able to support cell-to-cell interactions. However, while the proof of concept has been demonstrated that 3D matrices seeded with thymic stromal cells can partially support T cell development from precursor hematopoietic cells (180, 181), the field has been limited by a lack of a sustaining source of epithelial cells to seed. However, recent studies have used several approaches that could overcome this barrier, including 1) identifying endogenous thymic epithelial progenitor cells (TEPC), 2) driving differentiation of embryonic stem cells or iPS cells into TEPC, and 3) transdifferentiation of other cell lineages into TEC-like, T cell supporting cells.

Although TEPC were identified in the developing thymus almost 15 years ago (182, 183), and the presence of an adult progenitor did indeed exist (184), it is only in the past two years that significant progress has been made into identifying the postnatal TEPC (185,186). Recent work has also demonstrated methods that could be used to drive primitive stem cells into thymic epithelial progenitors cells by precise regulation of crucial factors involved in TEC commitment and maturation, including FOXN1, HOXA3, TGFβ, BMP4, Wnt, Shh, and FGF signaling (187–190), which could be a promising source of cells for a bioengineered thymus. Finally, an innovative alternate approach was taken to generate TECs by direct reprogramming of mouse embryonic fibroblasts (MEFs) through the forced expression of FOXN1 (191). These FOXN1-induced TECs (iTECs) promote T cell development in vitro and in vivo when transplanted into nude mice and could be used to seed an artificial bioengineered thymus.

Another technique that may hold potential to generate organs in vitro is based on tissue decellularization methods. In this technique, all cells are removed from the organ, leaving the extracellular matrix intact. It has been reported that decellularization of the thymus, followed by reconstitution with thymic stromal cells and lineage negative BM progenitors, led to formation a functional thymus when transplanted into the kidney capsule of nude mice (192).

Conclusions and future directions

The thymus is an outlier, which behaves out of keeping with many other organs. Extremely sensitive to acute damage, it can rapidly involute, but it initially has immense ability to recover from such insults. However, with age, this ability to regenerate is lost, and in fact aging of the thymus proceeds at a faster rate than other tissues. A greater understanding of these processes, in close collaboration with other branches of regenerative medicine and immunology, will pave the way to new therapeutic options. Indeed, such work has already led to the establishment of clinical trials examining cytokines, growth factors and hormonal

therapy (Table 1). The challenge for the future is to continue development of new strategies, and to ensure that those with potential undergo rigorous evaluation in the clinical environment.

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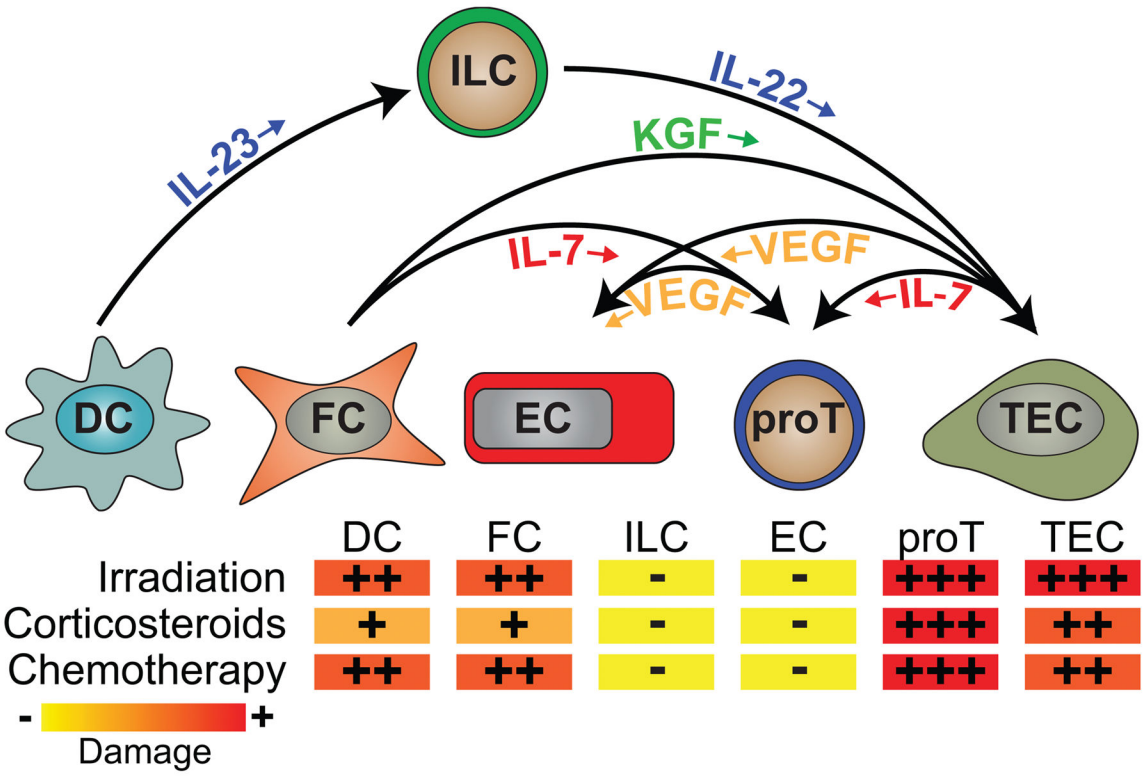

Figure 1. Targets of acute thymic damage and pathways of endogenous regeneration

The thymus is extremely sensitive to damage, typically in the form of irradiation, cytoreductive chemotherapy or stress-induced (or administered) corticosteroids. While most of these insults target the T cell progenitors (most prominently CD4+CD8+ DP thymocytes), TECs are also notably targeted by both irradiation and cytoreductive chemotherapy. Corticosteroids specifically target thymocytes and so other cell populations including TECs, dendritic cells (DCs), fibroblasts (FC), innate lymphoid cells (ILCs) and endothelial cells (ECs) are relatively untouched initially (although due to crosstalk there is a decline in the numbers of cTECs and mTECs after the thymocyte depletion). ILCs and ECs, and to a lesser extent FC and DC, are remarkably resistant to acute damage. After injury the thymus has a remarkable capacity to regenerate itself. While the mechanisms underlying this regeneration remain poorly understood, in the past few years several pathways have been revealed. These include the IL-23/IL-22 and KGF pathways, which targets TECs; IL-7, which can be produced by both TECs and FCs and target early T cell progenitors; and VEGF, which can be produced by TECs and some thymocytes and targets ECs to induce angiogenesis, a crucial step during organ regeneration.

Table 1

Strategy	Regenerative Targets	Stage of Development	References
IL-7	BM HSPCs Thymocytes	In trials	(136, 138, 141, 193–197)
IL-12	Thymocytes	Pre-clinical	(198, 199)
IL-21	Thymocytes	Pre-clinical	(200)
IL-22	TECs	In trials	(19)
Flt3L	BM HSPCs Thymocytes	Pre-clinical	(201–205)
IGF-1	TECs	Pre-clinical	(158, 206)
GH/Ghrelin	Thymocytes	In trials	(207, 208)
KGF	TECs	In trials	(68, 69, 126–129)
SCF	Thymocytes	Pre-clinical	(209)
Sex steroid inhibition	TECs BM HSPCs Thymocytes	In trials	(6, 36–38, 76, 164–166, 210)
Precursor T	Thymocytes	IND pending	(174, 176, 211)
HSPCs	Thymocytes	Pre-clinical	(212)
ex-vivo TECs	TECs Thymocytes	Pre-clinical	(187, 190, 191, 213, 214)
Thymus bioengineering	Mature T cells	Pre-clinical	(192, 215)