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Rejuvenation of the Thymus in Humans Post Adolescence

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ABSTRACT

T lymphocytes, an indispensable component of the adaptive immune system, protect our bodies against infections, tumors etc. T cell progenitors are produced in the bone marrow and their sequential differentiation occurs in the thymus. The most immature cells, CD4-CD8- thymocytes give rise to the CD4+CD8+ cells. These cells undergo positive and negative selection, after which only the non-self-reactive and immune competent T cells survive. Subsequently, the phenotypically and functionally mature CD4+CD8- or CD4-CD8+ T cells egress from the thymus into the periphery as recent thymic emigrants. For constant efflux of selected thymocytes with a diverse T cell receptor repertoire into the periphery, stable functioning of the thymus is necessary. However, the thymus undergoes reduction in its cellularity, known as thymic atrophy, due to its exceptional sensitivity to stress and other factors. Thymic atrophy in humans occurs physiologically with ageing and pregnancy, and during stress conditions, including malnutrition, infections, cancer chemotherapies etc. Thymic atrophy is also observed in patients with graft-versus-host disease, Down's syndrome and sudden infant death syndrome. Thymic output reduces with age and it is perceived to be of lesser significance later in life. However, studies have revealed that the thymus continues to function in older people. In fact, thymic functioning is crucial in scenarios post transplantation, chemotherapy and antiretroviral therapy. This review focuses on conditions where the human thymus atrophies and interventions (e.g. supplementation of thymulin, antioxidants, IL-7, growth hormone, ablation of androgens, etc.), including clinical trials, which rescue thymic cellularity and/or enhance thymic output.

Keywords: IL-7, T cell development, Sex steroids, Thymus, Thymic atrophy, Thymulin

Abbreviations: aGVHD: Acute Graft-Versus-Host Disease; AIRE: Autoimmune Regulator; BMT: Bone Marrow Transplantation; cTECs: Cortical TECs; Cy: Cyclophosphamide; Dll4: Delta-like 4; DN: Double Negative; DP: Double Positive; ETP: Early Thymic Progenitors; FSP: Fibroblast Specific Protein 1; GC: Glucocorticoid; GH: Growth Hormone; GVHD: Graft-Versus-Host Disease; HAART: Highly Active Antiretroviral Therapy; HSCT: Hematopoietic Stem Cell Transplantation; IL-7R: IL-7 Receptor; ISP: Immature Single Positive; KGF: Keratinocyte Growth Factor; LHRH: Luteinizing Hormone-Releasing Hormone; mTECs: Medullary TECs; RTE: Recent Thymic Emigrants; sjTREC: Signal Joint TCR rearrangement Excision Circles; SP: Single Positive; TCR: T Cell Receptor; TEC: Thymic Epithelial Cells; TRA: Tissue-Restricted Antigens; TREC: TCR Rearrangement Excision Circles

INTRODUCTION

T cell development, selection and maturation occur in the thymus, a primary lymphoid organ. The thymus was named by Galen (129-210 or 216 AD) because of its structural resemblance to the leaf of the thyme plant [1]. For years it was considered to be a vestigial organ till it was reported as the site of T cell development, making it one of the last major immune organs to be discovered [2,3]. Evolution suggests that it is one of the newer organs to appear, observed for the first time in fish [4]. The thymus is highly conserved in terms of developmental origin, anatomical location and function in all jawed vertebrates, i.e., gnathostomes [5]. Interestingly, in the jawless fish lampreys, primitive thymus-like lympho-epithelial structures called thymoids are found. These structures express the transcription factor, FOXN4, the ortholog of forkhead

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boxN1 (FOXN1), which is essential for differentiation of thymic epithelium in jawed vertebrates [6]. In fact, the emergence of these rudimentary structures in lampreys coincided with the emergence of the two arms of adaptive immunity, cellular and humoral [7].

T cell development occurs via cell-cell interactions between thymocytes and cells that constitute the stromal cell network, e.g. thymic epithelial cells (TECs), dendritic cells, B cells and macrophages. T cell progenitors originating from the bone marrow arrive into the thymus as early thymic progenitors (ETPs). The development of T cells can be monitored using cell surface expression of the T cell co-receptors, CD4 and CD8, wherein CD4-CD8- (double negative, DN) are the most immature thymic subset, that give rise to the CD4+CD8+ cells (double positive, DP) via an intermediate cell population, the immature single positive cells (ISP) **(Figure 1)**. T cell selection in the thymus is spatially compartmentalized and is executed primarily by the TECs, wherein the cortical TECs (cTECs) and the medullary TECs (mTECs) mediate positive and negative selection respectively. During positive selection, the DP thymocytes that express the T cell receptor (TCR) are capable of recognizing self-peptides on cTECs receive survival signals and develop into CD4+/CD8+ single positive (SP) thymocytes. More than 90% of thymocytes do not get positively selected and undergo apoptosis. The majority of negative selection occurs on SP thymocytes and is mediated by mTECs which express a wide variety of tissue-restricted antigens (TRA). The developing thymocytes that are reactive to self-antigen-MHC complexes are eliminated, thus preventing the emergence of autoimmune T cells. The expression of TRA is regulated by a transcription regulator, the autoimmune regulator (AIRE), which is expressed in ~30% of mTEC and a subset of B cells. AIRE-deficient mice display decreased TRA expression and autoimmunity including autoantibody production and autoimmune T cells in the periphery [8,9]. Following negative selection, the SP cells mature and the cells egress from the thymus as phenotypically and functionally competent naïve cells, knows as recent thymic emigrants (RTEs) **(Figure 1)**. The fitness or the output of the thymus is determined by the number of RTEs in the periphery. Quantification of signal joint TCR rearrangement excision circles (sjTREC) is one of the most commonly used methods of measuring RTEs. sjTRECs are circular extra-chromosomal by-products generated during the TCRα chain rearrangement. These DNA circles do not replicate and thus their frequency reduces with every cell division [10]. Apart from sjTRECs, in humans CD31 (PECAM-1), a member of the Ig superfamily, is used to quantify RTEs. Human RTEs are CD31+, contain sjTRECs and express CD45RA. Peripheral expansion of RTEs give rise to central naïve T cells that lack sjTRECs and only express CD45RA but not CD31 [11]. Contrary to popular belief, sjTREC studies in humans have demonstrated that the thymus is active throughout life, with the output of the thymus declining abruptly only in the 10th decade [12].

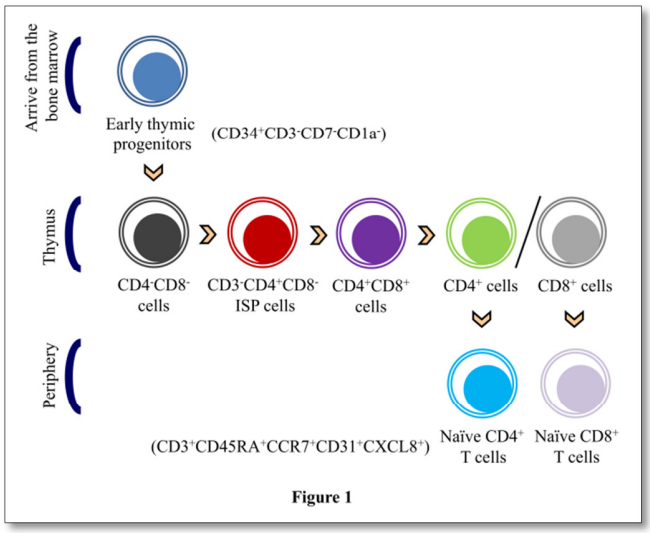

Figure 1. A simplified diagram of T cell development in humans.
The progenitor T cells arise in the bone marrow and arrive to the thymus as early thymic progenitors (ETPs). Subsequently a sequential and multistep process follows wherein the thymocytes modulate various cell surface markers, thymic selection occurs and immunocompetant, non-self-reactive naïve T cells are generated. At the CD4-CD8- double negative (DN) stage, the rearrangements of DJ at the TCRβ start, while the VDJ TCRβ rearrangements are initiated at the CD3-CD4+CD8- immature single positive cell (ISP) stage. Following transition to CD4+CD8+ double positive (DP) stage, the thymocytes having undergone successful TCRβ gene rearrangement, downregulate recombination-activating genes (RAG) expression and β-selection ensues. Clonal proliferation follows and RAG genes are re-expressed for TCRα gene rearrangement. Positive and negative selection occurs to eliminate the self-reactive thymocytes and to select for the immunocompetent T cells. As the thymocytes develop, they migrate from the cortex towards the medullary region of the thymus. The DP cells give rise to either the CD4+ or the CD8+ single positive (SP) cells. Following maturation, cells emigrate the thymus as naïve T cell. In case of humans, the most widely used markers to identify naïve T cells and thus quantitate thymic output are CD31 and TRECs [82]

The thymus is one of the most sensitive organs to atrophy, i.e., loss in thymic cellularity and its architecture and output. It is widely perceived that the activity of the thymus is known to reduce starkly post puberty and as a consequence is not beneficial further in life. Although the peripheral naïve T cell pool in humans is maintained almost independent of the thymus post adolescence [13], the thymus continues to remain active, and its functioning is vital for T cell

reconstitution post highly active antiretroviral therapy (HAART) in HIV-infected patients, bone marrow transplantation (BMT) and chemotherapy [14]. Recently an immunological model has proposed that there is a strong correlation between incidences of infectious diseases and cancer and T cell output, making thymic atrophy a significant risk factor [15]. This review aims to discuss the conditions in which the human thymus atrophies followed by therapeutics to rejuvenate it. We have also cited the most recent developments in the field using animal models wherever necessary.

THYMIC ATROPHY IN HUMANS

Thymic atrophy often results in reduced thymic output and naïve T cell numbers in the periphery along with a restricted TCR repertoire. These may result in dampened responses to novel pathogens, reduced T cell reconstitution post transplantation, poor response post vaccine challenge and decreased tumor surveillance [16]. Thymic atrophy is well known to physiologically occur during ageing and pregnancy and also during myriad clinical conditions. Many factors, listed elsewhere [14], can either independently or in concert cause thymic atrophy.

Some of the instances where the human thymus undergoes atrophy or its output reduces are as follows: Cortisol, secreted by the adrenal cortex, is the primary glucocorticoid (GC) produced by the body. GCs induce thymocyte apoptosis in a calcium-, ATP- and caspase-dependent manner [17]. Astronauts returning from space flights had elevated amounts of cortisol in urine and plasma and reduced thymic output as measured by TREC content [18]. Thymic damage can also result in cases including acute graft-versus-host disease (aGVHD). Allogeneic hematopoietic stem cell transplant (HSCT) can lead to aGVHD, which increases the risks of chronic GVHD, a major factor causing morbidity and mortality in BMT recipients [19]. aGVHD reduces the cellularity of the intrathymic $AIRE^{+}mTEC^{high}$ cells, resulting in emergence of autoimmunity [20]. Severe reduction in thymic output as measured by sjTREC content is also observed in patients of Down's syndrome, which is caused due to an autosomal disorder [21]. Thymus transcriptome studies have revealed that the reduced thymic output is due to the hypoexpression of genes related to antigen processing and presentation, T cell differentiation and selection and AIRE-partner genes and not due to premature ageing as previously perceived [22]. Another example of thymic atrophy is sudden infant death syndrome (SIDS), which is the leading cause of infant death within the first year of life. Exogenous stressors are hypothesized to contribute towards the phenomenon and the health of the thymus was considered as a parameter in a study. Thymic from SIDS infants displayed reduced proliferation of thymocytes and enhanced macrophage activity, hallmarks observed during stress-induced inflammation [23].

THERAPEUTICS KNOWN TO REJUVENATE THE THYMUS IN ADULTS

Thymulin

Thymulin, the thymic peptide hormone, is secreted by TECs [24]. Zinc acts as thymulin's cofactor, making its presence indispensable for the peptide hormone's activity [25]. In malnourished children, zinc supplementation augments their thymus size [26], possibly by increasing the levels of active thymulin. With ageing, the zinc pool progressively depletes in humans, which may contribute towards age-associated thymic atrophy due to reduced activity of thymulin as demonstrated in old mice [27]. Extra thymic production of thymulin is reported in macrophages and fibroblasts under stress conditions including heat, oxidative stress, apoptosis and necrosis [28].

Acute zinc deficiency is found in patients infected with HIV at different stages of the disease [29]. In fact, the zinc-bound active form of thymulin is very low or undetectable in HIV-positive pediatric patients who progress to AIDS [30]. Zinc deficiency and low CD4+ T cell counts are significant risk factors towards incidence of opportunistic infections in HIV-infected patients. Accordingly, monitored supplementation of zinc in the diet of late stage HIV-positive patients along with HAART leads to complete reduction of infections by *Candida aesophagea*, *Pneumocystis carinii*, etc. [31]. Long-term zinc supplementation in the diet delays immunological failure and reduces diarrhea by more than half in HIV-infected adults [32]. In addition, keeping the above mentioned studies into perspective, the widely reported thymopoietic properties of growth hormone [33,34] may be due to its ability to increase thymulin secretion [35].

IL-7

IL-7 is a non-hematopoietic cell-derived, non-redundant lymphopoietic cytokine. Its roles in T cell development are evolutionarily conserved, from lower vertebrates to humans. Mutations in the IL-7 signaling pathway acutely affect thymopoiesis in zebrafish [36]. A homeostatic mechanism exists to keep the IL-7-expressing cells in check. After positive selection into the CD4 lineage, the frequency of IL-7-expressing TECs reduces moderately, whereas negative selection results in a prominent loss of these cells [37]. IL-7 is required during various stages of T cell development, maturation and survival and its multifaceted functions are summarized in **Table 1**.

Table 1. Distinct functions of IL-7 during T cell development.

Cell population/s	Functions
DN	IL-7 promotes survival and development by phosphorylating and activating the lymphoid-specific transcription factor, NFATc1 in these cells [83].
γδ T cells	In IL-7R-deficient mice, the V-J recombination of the TCR gamma genes is specifically blocked, leading to impaired expansion of early lymphocytes and the lack of γδ T cells [84].
ISP	IL-7 promotes the survival of these thymocytes [85].
DP	IL-7R enhances the anti-CD3 activated Akt, STAT5 and Erk1/2 signalling pathways, thus synergistically augmenting pre-TCR signalling [86].
Thymic regulatory T cells (tTregs)	IL-7 enhances the survival and upregulates the expression of Foxp3 and CTLA-4 in Treg cells at the DP stage, thus increasing its frequency [87].
DP – SP	IL-7 signalling mediates "coreceptor reversal" during which the developing DP thymocytes downregulate CD8 transcription only to subsequently reinitiate it when cells differentiate into CD8+ SP cells [88].
CD8+ SP	IL-7 is required for differentiation of cells to the MHC class I-restricted CD8+ T cell lineage [89].
SP cells	IL-7R regulates development and maturation of SP cells as IL-7R conditional knockout mice display reduced proportion and proliferation of CD4+ and CD8+ SPs [90].
RTEs	IL-7 in low doses and at short durations, promotes cell survival, while in higher doses with continuous exposure, induces proliferation. The former event is mediated by STAT5 tyrosine phosphorylation, while the latter increases glucose uptake and upregulation of the glucose transporter, Glut-1 via the phosphoinositide 3-kinase pathway [91].
T cell activation and survival	IL-7 regulates basal T cell metabolism, as deletion of the IL-7R leads to reduced T cell survival, impediment in mitogenesis and decreased glycolysis along with delayed proliferation and growth post stimulation [92].
Peripheral regulatory T cells (pTregs)	IL-7 regulates the homeostasis of these cells and upregulates Foxp3 [93].

Defective IL-7 receptor (IL-7R) signaling is observed in patients suffering from severe combined immunodeficiency [38,39]. Strikingly, IL-7R deficiency in mice causes absence of both B and T cells, while in humans the B cells are present [38,39]. Continuous thymic activity is required for IL-7-mediated proliferation of naïve CD4+ T cells. Dampened IL-7-driven homeostatic proliferation is observed in CD31+ naïve CD4+ T cells from individuals thymectomized in early childhood during corrective cardiac surgery [40]. There are numerous factors which contribute to IL-7-mediated thymopoiesis **(Table 1)**.

IL-7 is expressed by TECs, which supports survival and maturation of thymocytes [41]. Studies in mice have demonstrated that the frequency of cells expressing high amounts of IL-7 reduces with age, probably contributing to age-associated thymic atrophy [42]. Administration of IL-7 directly increases TREC content in adult as well as in fetal thymus, possibly due to increased TCR rearrangement [43], although contradictory observations have also been reported in ageing mice [44]. Interestingly a correlation of physical activity to thymic output has been recently reported. Older adults who maintained a high level of physical activity

(cycling) had comparable levels of naïve T cells and RTE to that of young adults. Their age-matched less physically-active counterparts had lower serum levels of thymopoietic hormones, IL-7 and higher levels of IL-6, which is known to induce thymic atrophy [45].

Lower mortality in Gambian infants born in the harvest season compared to those born in the hungry season has been associated with higher amounts of IL-7 in breast milk, which may be responsible for increased thymic index and higher sjTREC amounts in peripheral T cells [46]. IL-7 is present in maternal milk and is capable of crossing the gut. Il-7-deficient mice develop lymphopenia and Il-$7^{-/-}$ pups when fed milk from wild type mice display increased thymic and splenic cellularity [47].

In the first human clinical trial for IL-7, recombinant human IL-7 upregulated Bcl2, induced cycling and expansion of peripheral T cells including the naïve T cell compartments and enlarged the TCR repertoire [48]. Moreover, a recombinant human IL-7 broadened the TCR diversity and enhanced the effector memory cells in a clinical trial consisting of patients which underwent T cell depleted allo-HSCT [49]. Co-transduction of BM-derived mesenchymal stem cells with two reported thymopoietic factors, IL-7 and stem cell factor has been demonstrated to synergistically induce thymopoiesis and aid in T cell reconstitution post BMT in mice [50]. Successful thymopoiesis as well as expansion and survival of T cells in the periphery are observed in mice treated with IL-7 post BMT, indicating T cell reconstitution by IL-7 is both thymus-dependent as well as thymus-independent [51,52]. However, some contradictory results also exist. For example, allogeneic HSCT patients with elevated IL-7 levels display increased severity of aGVHD and reduced number lymphocytes and overall lifespan [53]. Also, blockade of the IL-7R post T cell depletion with skin allografts diminishes cellular and humoral responses and enhances the graft survival in mice [54].

IL-7 therapy has been demonstrated to be harmful in HIV-infected patients. Plasma IL-7 levels are elevated in HIV-infected patients and it is speculated to work in a feedback mechanism to restore peripheral T cell numbers [55]. However, it is suggested that IL-7 when administered in HIV-infected patients on HAART, results in 70% increase in the number of circulating CD4+ T cells which contain integrated HIV DNA. This increase in the number of T cell is not thymus-driven, but due to enhanced T cell cycling and survival [56]. Therefore, IL-7 increases the persistence of HIV [57]. Elevated IL-7 levels at late stages of HIV disease progression [58] induces the expression of cell surface CXCR4 on CD4+ T cells [55,59], resulting in a switch of HIV-1 co-receptor tropism from CCR5 to CXCR4 [60], which may accelerate disease progression [61]. In addition, *in vitro* studies demonstrate IL-7-mediated STAT5 phosphorylation and Bcl2 expression are down regulated during HIV infection in thymocytes [62]. In corroboration, HAART in HIV-infected patients is successful in boosting thymic functions, while intrathymic IL-7 amounts are reduced [63], indicating an inverse correlation between output of the thymus and IL-7 levels. Further studies are required to fully understand the roles of IL7 as a prospective thymopoietic agent.

SEX STEROID ABLATION

Sexual dimorphism in the thymus and thymic output has been documented in several studies. In a couple of hypogonadal men, the numbers of naïve CD4+ T cells, i.e., CD45+CD4+ were found to be greatly increased. Importantly in these patients, the TREC amounts reduced drastically post androgen replacement therapy [64]. One of the plausible reasons may be the reduction in the cellularity of ETPs with age, as it can be ameliorated by castration. Post androgen withdrawal in mice, the rise in ETPs and eventual enhancement of thymopoiesis is mediated via increased proliferation of TECs and production of CCL25, the CCR9 ligand, which is crucial for ETP immigration into the thymus [65]. In male mice, cTECs are more abundant than in females, although these cells display reduced proliferation and low expression of FoxN1 and its target genes. In addition, the cTECs in males express lower levels of genes crucial for thymocyte development and selection such as Psmb11 (a cTEC-specific proteasome subunit), Ctsl (a peptidase crucial for positive selection of CD4+ SPs) and the Notch ligand, Delta-like 4 (Dll4) [66]. Dll4 is indispensable for T cell lineage commitment to occur and its absence leads to emergence of immature B cells in the thymus. Inhibition of Dll4 in cTECs by testosterone abrogates thymopoiesis in mice. Also, chemical castration by a luteinizing hormone-releasing hormone (LHRH) antagonist results in higher expression of Dll4 [67].

The existence of sex-associated differences in the expression of TRA has been reported, which culminate in higher susceptibility to autoimmune diseases in females than in males. This is primarily due to reduced expression of AIRE in both mice and human thymic post-puberty in females. Accordingly, castration in male mice reduces AIRE expression [8]. Androgen recruits the androgen receptor to the AIRE promoter regions and upregulates its transcription. This has been demonstrated in a mouse multiple sclerosis model where androgen treatment and the male gender confer AIRE-dependent protection against experimental autoimmune encephalitis [68]. On the other hand, estrogen treatment downregulates AIRE in cultured human TECs and human thymic implants in immunodeficient mice as well as in fetal thymus organ culture. Moreover, estrogens induce epigenetic changes in females, as the number of methylated CpG sites on the AIRE promoter are upregulated, thus reducing AIRE expression [8].

Not surprisingly, sex steroid inhibition has been exploited to rejuvenate the thymus. In aged prostate cancer patients

treated with localized radiation and upon temporary chemical castration using a LHRH agonist treatment increases the CD4+ and CD8+ T cells (naïve and memory cells) in the periphery, along with enhanced TREC contents in the majority of the patients [69]. Similarly, the pre-treatment of a LHRH agonist to HSCT patients augments the naïve CD4+ T cell numbers, the diversity of the TCR repertoire and the TREC levels in the CD4+ T cells [70].

Cancer therapies including anti-cancer agents also lead to thymic atrophy. During chemotherapy in testicular cancer patients, the thymus atrophies [71]. In a study, 90% of the patients with metastatic diseases displayed reduction in volume of the thymus during chemotherapy and its rejuvenation occurred during the recovery phase [72]. Mice treated with anti-cancer agents such as cyclophosphamide (Cy) develop thymic atrophy [73-75]. Cy treatment depletes the thymic CD45- fibroblast specific protein 1 (FSP1)+ cells. The thymic FSP1+ fibroblasts *in vitro* release growth factors important for TEC proliferation including FSP1, IL-6, keratinocyte growth factor (KGF) compared to FSP1 negative cells. Mice deficient in FSP1 expression display reduced mTECs and severe thymic atrophy [74].

OTHER THERAPIES

The thymus is required for immune reconstitution post HAART in HIV-infected patients. Growth hormone has been successful in increasing the density of the thymus along with enhancing the TREC frequency in PBMCs as well as the naïve T cell counts [33]. The antioxidants, Vitamin C and N-acetyl cysteine have also demonstrated probable thymopoietic effects by increasing the CD4+ T cell numbers along with reducing the HIV RNA plasma levels in HIV-infected patients [76].

On the other hand, despite showing promise as a thymopoietic agent in animal studies [14], recombinant human KGF administration is unsuccessful in augmenting the thymic output HIV-1 infected patients [77]. It will be worthwhile to consider the results of the ongoing clinical trial on the effect of KGF in enhancing thymic reconstitution and reducing the occurrences of autoimmune diseases in multiple sclerosis patients being treated with a humanized IgG1 monoclonal antibody that targets CD52 [78].

CONCLUSION

The importance of the thymus post adolescence is vastly underappreciated. As a result, there is a lack of studies which focus on the effect on the thymus during various ailments and interventions to counter them. This review exclusively focuses on conditions during which the human thymus atrophies and interventions to dampen the process **(Figure 2)**. Most of the studies on the thymus or its activity are performed on animals including rodents, due to the shortage of thymus specimens available, with the only sources being Myasthenia gravis patients and subjects undergoing cardiac surgery [14]. Thus, the thymopoietic drugs demonstrating potency in animal studies may not necessarily translate to humans, such as the failure of KGF and IL-7 under certain conditions. Many clinical trials have been performed or are ongoing which evaluate the efficacy of drugs for thymopoietic potential. Trials such as monitoring the thymic size and output upon androgen blockade therapy for prostate cancer in older patients were terminated due to low accrual (NCT00379119). However, the effect of growth hormone on the thymic function in HIV-infected adults (NCT00379119) was found to be successful in increasing the thymic output [79]. During an antiretroviral therapy trial, HIV-infected children were shown to interrupt the decline of CD4+ T cells with early antiretroviral therapy. The reconstitution of CD4+ T cells was proportional to the thymic output [80]. Some of the ongoing clinical trials include the assessment of the efficiency of PET/CT scan and MRI to quantify the thymic size and function (NCT02909075). A phase 2 clinical trial is active to check for the efficacy of the androgen blocker, Lupron for immune reconstitution, reduction in GVHD and infection in recipients of allogeneic BMT (NCT01338987). Further studies are required which employ parameters such as CD31 and sjTREC levels to monitor the thymic activity during various conditions in humans. Screening and rigorously testing of molecules proven to be thymopoietic either alone or in concert in animal models [81] can be further considered as novel candidate therapies in humans.

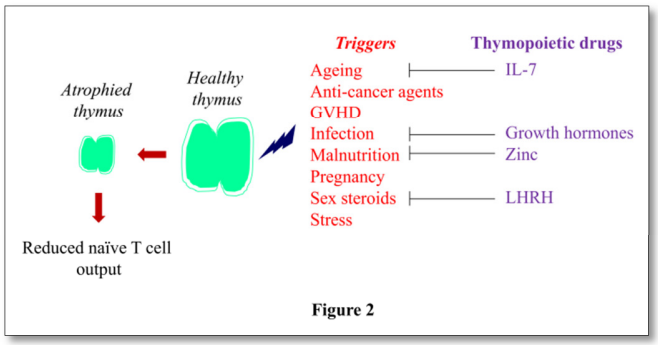

Figure 2. Thymopoieitic drugs to counter thymic atrophy in humans.
Although the cellularity of the thymus reduces sharply post adolescence, thymic output is detected throughout life. Thymic activity is required for T cell reconstitution post transplantation, anti-cancer and anti-viral therapy. Triggers resulting in thymic atrophy in humans and the limited agents known to demonstrate thymopoietic potential are depicted in the figure

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

DN conceived the idea. SM and DN wrote and approved the final version of the manuscript.

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