



Dynamics of thymus function and T cell receptor repertoire breadth in health and disease

David Granadier^{1,2,3,4} · Lorenzo Iovino^{1,2} · Sinéad Kinsella^{1,2} · Jarrod A. Dudakov^{1,2,5} 

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Abstract

T cell recognition of unknown antigens relies on the tremendous diversity of the T cell receptor (TCR) repertoire; generation of which can only occur in the thymus. TCR repertoire breadth is thus critical for not only coordinating the adaptive response against pathogens but also for mounting a response against malignancies. However, thymic function is exquisitely sensitive to negative stimuli, which can come in the form of acute insult, such as that caused by stress, infection, or common cancer therapies; or chronic damage such as the progressive decline in thymic function with age. Whether it be prolonged T cell deficiency after hematopoietic cell transplantation (HCT) or constriction in the breadth of the peripheral TCR repertoire with age; these insults result in poor adaptive immune responses. In this review, we will discuss the importance of thymic function for generation of the TCR repertoire and how acute and chronic thymic damage influences immune health. We will also discuss methods that are used to measure thymic function in patients and strategies that have been developed to boost thymic function.

Keywords Thymus · TCR repertoire · Damage and regeneration

Introduction

The adaptive immune system relies on a diverse repertoire of receptor bearing lymphocytes allowing for the recognition of an untold number of potential pathogen targets and for the surveillance of cancerous mutated self-antigens. The integrity of the thymus is essential for the output of T cells with diverse

receptors, a point most dramatically demonstrated by the immunodeficiency in patients with DiGeorge syndrome (DGS)—a profound inborn immunodeficiency in which a 22q11 deletion results in defective third and fourth pharyngeal pouch development and thus hypoplasia of the thymus. However, even in healthy individuals, thymic function is a dynamic process with tissue function severely impacted by negative stimuli. These can be broadly separated into two categories, with different outcomes on immune health and ultimately different clinical parameters for therapeutic intervention. The first of these are the acute injuries such as everyday insults like stress and infection, but also more profound injuries such as that caused by common cytoreductive cancer therapies. The second category encompasses chronic damage such as age-related thymic involution, persistent infection, and chronic stress. In this review, we will discuss these different injuries to the thymus and their influence in disease pathophysiology, and discuss preclinical and clinical evidence on potential therapeutic strategies to boost thymic function.

David Granadier, Lorenzo Iovino and Sinéad Kinsella contributed equally to this work.

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✉ Jarrod A. Dudakov
jdudakov@fredhutch.org

- ¹ Program in Immunology, Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA
- ² Immunotherapy Integrated Research Center, Fred Hutchinson Cancer Research Center, Seattle, WA, USA
- ³ Medical Scientist Training Program, University of Washington, Seattle, WA, USA
- ⁴ Department of Molecular and Cellular Biology, University of Washington, Seattle, WA, USA
- ⁵ Department of Immunology, University of Washington, Seattle, WA, USA

Acute damage: restoring T cell numbers

Everyday insults: hormones and infection

Stressors leading to rises in systemic cortisol, a glucocorticoid hormone, are well known to cause thymic involution via

apoptosis of thymocytes and clinical studies show a negative relationship between systemic corticosteroid levels and thymic function [1]. Glucocorticoids are central to many acute forms of thymic involution [2], directly inducing apoptotic cell death of CD4+CD8+ DP thymocytes, which preferentially express the glucocorticoid receptor; the same effect also occurs with commonly used glucocorticoid immunosuppressives [3].

Increases in sex hormones also contribute to thymic injury both in the acute and chronic setting [4] and are even thought to play a role in age-related thymic atrophy and can directly induce thymic involution [5] (see below). During pregnancy, rises in sex hormones lead to acute thymic involution, a biologically useful process believed to be due, at least in part, to progesterone activation of osteoclast differentiation receptor (RANK) on thymic epithelial cells that cause reduction in normal thymocyte production and the expansion of regulatory T cells that aid in the prevention of miscarriage [6].

Although generation of new T cells and their export into the periphery is important for infection response [7, 8], most acute viral infections paradoxically result in acute thymic atrophy; largely due to intense lymphocyte depletion as a result of increased apoptosis of thymocytes and interference with thymocyte development [9]. Infection-related thymic involution can be partially attributed to rises in TNF α and increased production of IFN γ from activated CD8+ T cells and natural killer (NK) cells. Acute bacterial infections have also been implicated, with *Streptococcus suis* infection leading to thymic involution by triggering apoptosis in developing thymocytes [10]; while *Mycobacterium tuberculosis* infection lead to thymic atrophy [11], though this is likely primarily mediated by glucocorticoids [12].

In all studies that have explored thymic involution in response to acute infection and/or stress, the thymus is capable of remarkable repair and rejuvenation. Therefore, everyday insults such as stress and infection are not thought to have a prolonged impact on thymic function; although there are unanswered questions to the long-term impact of repeated minor acute insults.

Profound acute damage: cytoreductive therapies

In addition to the everyday insults like stress and infection, the thymus is also exquisitely sensitive to cytoreductive therapies like chemotherapy and radiation, often used in the conditioning required for successful hematopoietic cell transplant (HCT) [13, 14]. In mouse models, studies have found that after both total body irradiation (TBI) or chemotherapy, in addition to the direct depletion of highly proliferative thymocytes, there is significant damage to the nonhematopoietic epithelial microenvironment resulting in reduced T cell development [15, 16]; which may result from the relatively high rate of turnover of some TEC subsets [17]. Although damage

and recovery are worse in older individuals whose thymus has already undergone significant involution, prolonged T cell depletion after cytoreductive therapies can be dangerous in even relatively young individuals; which has been shown in cohorts of human patients [18] and in mice [19, 20]. In long-term follow-up studies of patients receiving allogeneic-HCT, delayed T cell reconstitution can last a year or more due to a delay in full recovery of T cell numbers, and is associated with increased risk of infections, relapse of malignancy, and the development of secondary malignancies [21–28].

Notably, the thymus is not only sensitive to the conditioning regimes required for HCT, but it is also extremely sensitive to the treatments used to suppress the immune system from the impacts of graft versus host disease (GVHD), as well as GVHD itself, as demonstrated in several studies on mouse [3, 29–33]. Furthermore, animal models have also helped in identifying a link between acute GVHD-mediated thymic damage and the formation of chronic GVHD, which may be due to a failure of tolerance induction [34–36].

Chronic insult: dynamic thymic function and TCR repertoire breadth

Chronic infection

Most of the studies evaluating thymic function in the context of chronic insult have concentrated on chronic infections such as HIV, which leads to several modes of thymic dysfunction including thymic atrophy, reduced thymic output, reduced export of immature thymocytes and disruption of the thymic microenvironment [37–39]; and *Cytomegalovirus* (CMV), which notably leads to overgrowth and clonal dominance of the peripheral repertoire [40, 41]. Notably, effective response to antiretroviral therapies was found to depend on competent thymic function, with enhanced function in HIV-infected children with higher basal levels of thymic function [42], in contrast with infected adults who have a reduced thymic output and output decreased peripheral CD4+ T cells [43, 44]. Moreover, in addition to viral load, quantification of CD4+ recent thymic emigrants (RTEs) has long been employed as a marker for HIV disease progression, and a recent study has demonstrated the use of RTE CD4+ T cells as a marker of perinatal HIV infection in infants [45]; further strengthening the link between viral infection, efficient thymic function and therapeutic implications of thymic recovery. Notably, dominance of virus antigen-specific T cell clones is found in several chronic models of persistent antigen stimulation, such as CMV [46], Epstein-Barr virus [47], and HBV [48], though the contribution of chronic infection induced reduction in thymic production relative to the peripheral expansion of virus-specific lymphocytes remains to be determined. Studies in SARS-Cov-2–

infected patients have suggested a link between COVID-19 disease severity and T cell counts, with a well-defined lymphopenia observed in hospital-admitted patients, but the mechanistic link is not yet clear [49].

Age

Age-related thymic atrophy, or involution, occurs in almost all vertebrates [50], and is characterized by the progressive regression of thymic size and structure, resulting in impaired thymopoiesis [51, 52]. The profound loss of thymic functional capacity that occurs from thymic atrophy is one of the most studied aspects of immune aging. In addition to global decrease in thymic output, the aged thymus outputs T cells that are functionally inferior to those exported from the young thymus, with the deterioration of their quality centered on disturbances in intracellular signaling pathways resulting in a lack of antigenic stimulation and cytokine production [51, 53, 54]. Thymic involution ultimately leads to reduced responsiveness to new antigens [41, 55], and contributes to immunosenescence, which is marked by reduced thymopoiesis accompanied by ineffective central tolerance [56, 57]. Several factors are believed to influence thymic involution, including fewer hematopoietic progenitor cells [58, 59], the effects of altered sex hormone levels with age [60, 61], and TEC-driven structural changes [62]. Of note, although the age-induced reduction in lymphoid progenitors can exacerbate thymic involution [63], it is the alterations in the thymic microenvironment that are ultimately most responsible for driving the age-related decline of T cells [64].

Overall, it is the current hypothesis that unlike the directly targeted death of thymocytes in acute damage, the loss of the cortico-medullary junction is preceded by structural damage in both cortical and medullary TECs, the loss of established thymic architecture and increases in fibroblast numbers TEC apoptosis in mouse models [17, 62, 65]. Moreover, TEC homeostasis is largely under the control of the TEC autonomous expression of the transcription factor forkhead box N1 (Foxn1), reduced expression of which is identified in the involuted thymus [66]. With the steadily expanding numbers of elderly adults, thymic decline and T cell deficiency represents a profound underappreciated clinical complication. The implications of thymic involution are heavily centered on hampered naïve T cell output leading to a constriction in TCR repertoire diversity, reducing the probability of a sufficient immune response [55, 67]. Specifically, reduced output of new naïve T cells, coupled with oligoclonal expansion of memory T cells, contributes to the constriction of TCR repertoire breadth with age [68]. Moreover, increased output of self-reactive T cells likely contributes to multiple age-related disorders, including peripheral and neurological autoimmune disease [69].

Endogenous thymic regeneration

Although the thymus is extremely sensitive to injury it also has a remarkable capacity for repair [70–72]. In fact, the general phenomena of endogenous thymic regeneration has been known for longer even than its immunological function [73, 74]. Even in the surgical setting, children who have undergone partial thymectomy exhibit significant rejuvenation of the remaining thymic tissue [70]. Thus, endogenous thymic regeneration is a critical process to restore immune competence following thymic injury; however, endogenous thymic regeneration can be a prolonged process and is an important clinical problem in older patients receiving common cytoreductive therapies and recipients of HCT [18, 21–24]. Of key importance, the underlying mechanisms controlling this process have been largely unstudied [63, 75].

Recent preclinical work in mice has identified several distinct pathways that underlie endogenous thymic regeneration (Fig. 2). In the first of these, damage to the thymus triggers the production of IL-23 by a population of thymic dendritic cells, which in turn initiates the production of IL-22 by innate lymphoid cells (ILCs) [71, 76]. IL-22 acts on epithelial cells, including TECs and mediates thymic repair [33, 77]. Receptor activator of nuclear factor kappa-B ligand (RANKL), which is expressed by multiple subsets in the thymus including $\gamma\delta$ T cells, ILCs, and positively selected thymocytes [78–80], is also increased in after injury caused by the cytoreductive conditioning required prior to HCT, suggesting that RANKL plays a role in endogenous regeneration of the thymus [71, 81]. RANKL, which is a member of the tumor necrosis factor (TNF) superfamily, is important during thymic development due to its ability to drive the differentiation of TECs and induce the expression of AIRE [80, 82, 83]; although absence of RANKL postnatally can be compensated for by other factors [84]. RANKL can also stimulate TEC proliferation as well as their production of IL-7 [85], and overexpression of the soluble RANKL decoy receptor OPG causes an enlarged thymus [78, 86, 87] and exogenous administration of RANKL improved medullary architecture in RANKL-deficient mice [88]. In a second endogenous pathway of regeneration, thymic damage initiates the production of bone morphogenic protein 4 (BMP4) by radio-resistant endothelial cells (ECs). BMP4 has been known to be important for thymus organogenesis [89–91], and in fact BMP4 is a crucial factor in the induction of TEC-like cells from pluripotent stem cells [92–94]. BMP4 is produced by endothelial cells (ECs) after acute injury and acts on TECs, primarily cTECs, and induces their expression of FOXN1 and its downstream targets such as DLL4 [95], which is central to thymic regeneration [61]. Finally, work has also revealed that keratinocyte growth factor (KGF), produced primarily by fibroblasts and thymocytes, is also important for the endogenous response after damage [96]. KGF stimulates TECs to

induce their proliferation and expansion [97], but can also protect TECs from damage caused by alloreactive T cells in mouse models of GVHD [98].

Measuring thymic function

While studies in mice have elucidated considerable insight into thymic function, it is a challenge to truly assess thymic function in the clinical setting. Oftentimes, surrogate measures are used, ranging from readily available clinical parameters such as absolute lymphocyte counts (ALC) and quantitative assessment of T cell subsets (CD4⁺ and CD8⁺ T cells, MAIT cells, $\gamma\delta$ -T cells), to more complex and less routine assays to measure thymic functional mass, recent thymic emigrants (RTEs) or T cell receptor repertoires.

Absolute lymphocyte count

In the context of HCT, ALC, which is a routinely obtained clinical parameter, has been shown to be predictive of survival and relapse after both autologous and allogeneic transplant [99–103]. However, in a setting such as age or chronic insult—where there is no acute depletion of the endogenous T cell pool, ALC is less informative for general immune health.

Imaging for functional tissue

Although surrogate readouts like ALC can give a rough assessment of lymphocyte status, true assessment of thymic function can only be gleaned by directly analyzing the tissue itself. Given the correlation between thymic size and thymic function, imaging techniques may be employed in attempt to estimate thymic function. The anterior mediastinum is largely occupied by the thymus, which is consequently accessible to ultrasound [104, 105]. The absence of the mediastinal thymic profile in a chest X-ray is a hallmark in the suspect of congenital immunodeficiency of the childhood [106]. More advanced imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) are used in the diagnosis of thymomas and the latter is the gold standard radiologic follow-up of thymic neoplastic epithelial lesions [107].

Rebound thymic hyperplasia is a well-known phenomenon among children receiving chemotherapy and is appreciable as a diffuse ¹⁸F¹⁸FDG uptake in the mediastinum on positron emission tomography (PET) [108]; this technique is also able to detect spontaneous thymic regeneration in some adult patients [109], but these imaging approaches have also proved sensitive enough to detect changes in thymic size caused by chemotherapy [110, 111]. Moreover, in patients with HIV, increased thymic volume measured by CT correlated with response to antiretroviral therapy and peripheral CD4⁺ counts

[112, 113]; and a recent trial demonstrated that low prenatal thymic volume < 32 weeks of gestation was predictive of spontaneous preterm delivery [114]. However, while it is possible to use imaging techniques to measure functional thymic tissue, due to its costs this is not a feasible approach for routine measurement.

Recent thymic emigrants

A more promising and tractable surrogate of thymic function is to quantify the number of circulating RTEs [115]. In mouse studies, this can be done by either intrathymic injection of a dye such as FITC (though the stress of which may affect thymic function), or by using a reporter mouse strain where a fluorescent tag such as GFP is inserted under the promoter for RAG2, a gene fundamental for T cell (and B cell) development [116, 117]. In this model, the RAG2 (and GFP) gene is no longer transcribed after the DN stage of T cell development but GFP protein remains present into the periphery and is diluted as naïve cells expand, therefore marking RTEs [118, 119].

However, while useful for measuring thymic function in a preclinical setting, these tools are not available for measuring thymic function in the clinical setting. The gold standard for measuring RTEs in humans is by analyzing T cell receptor excision circles (TRECs), circular molecules formed from DNA excised during formation of the T cell receptor (TCR) encoded region [120–122]: an intermediate rearrangement event in most developing T cells destined to express the $\alpha\beta$ TCR results in the deletion of the TCR δ gene that lies 5' to the TCR J α region, generating a δ Rec- ψ J α TREC (signal joint TREC, or sjTREC), thereby ensuring that α and δ TCR protein chains are never coexpressed on the same T cell. This “beta” TREC assay is the most commonly used in the clinical practice to assess the exact number of RTEs; however, in particular, conditions such as during immune reconstitution following bone marrow transplantation or during HIV infection, this parameter can be affected by events occurring in the periphery such as T cell proliferation [123, 124]. Thus, data of sjTREC quantification should be cautiously interpreted. An alternative assay is based on measurements of the early occurring, single-step TCRG gene rearrangement [125] which results in the formation of a V γ -J γ coding joint (CJ_{V γ -J γ}) and a V γ -J γ signal joint (SJ_{V γ -J γ}) on the corresponding excision circle (V γ -J γ TREC). From the difference between CJ_{V γ -J γ} and SJ_{V γ -J γ} levels, the number of cell divisions undergone can be calculated [126]. It has been demonstrated that this calculation is able to discriminate between intra- and extrathymic T cell proliferation [127].

Use of TRECs has been shown to correlate strongly with thymic function in settings of age and conditioning such as for HCT [121, 122], where TRECs have shown more rapid recovery in younger recipients and in recipients of conventional

grafts compared to T cell depleted grafts [128], while the occurrence of chronic GVHD significantly decreases TRECs [129]. Furthermore, low TREC values correlated strongly with severe opportunistic infections [128, 130]. Notably, TREC measurement is a part of neonatal screening for congenital immunodeficiencies and to measure immune reconstitution after bone marrow transplantation [27, 131, 132]. Approaches have been developed to crudely identify TREC-enriched populations of T cells by flow cytometry using markers such as CD31 on CD4+ (but not CD8+) T cells [133–135]. However, a heterogeneous proportion (15–60%) of CD31+ TREC+ T cells across individuals indicates that CD31 is not a robust marker for identification of TRECs+ T cells.

TCR repertoire

Given the difficulty in getting truly evaluating thymic function clinically, and that the primary role of the thymus is to generate a self-tolerant but diverse repertoire of T cell receptors that are capable of recognizing unknown antigens, ultimately obtaining an accurate representation of the peripheral TCR repertoire is of greatest utility when measuring thymic function. However, while RTEs are quantifiable, measuring the total number of unique T cell clonotypes in circulation is a far greater challenge. TCR repertoire breadth reflects both the capacity of the thymus to generate naïve T cells and the cumulative responses of T cells to antigen challenges in the periphery [40, 41], but the challenge of quantifying the absolute number of unique clones speaks to the tremendous breadth of diversity that a healthy thymus can output. Since there are a potential for 10^{14} unique TCR β chains and 10^9 distinct α chains [136], mathematical modelling has suggested an estimate of $\alpha\beta$ diversity of 2×10^{19} possible clones [137, 138]. However, the range of probabilities of TCR β chain possibilities based upon the number of insertions and likelihood of certain V(D)J combinations was recently calculated to be as low as 10^{-18} for the rarest clones and as high as 10^{-6} for the most likely [139]; and the probability of generating any individual TCR $\alpha\beta$ is $< 10^{-12}$ [138]. Different analyses from deep sequencing of the TCR β chain estimate the lower bound of peripheral clonotypes to be from 10^6 to 10^8 [140, 141]. Statistical modeling approaches are used to estimate the total number of TCR clones in a given individual, a necessity given the limited quantity of cells achievable by peripheral blood draw [142]. Several include methods derived from models enumerating species diversity in an ecosystem in what is known as the “unseen species problem” [140, 142, 143]. However, the computational challenge of estimating the repertoire size may also yield considerable bias and is almost impossible to determine in individuals with perturbations in thymic function [144].

Many of the experimental estimates for TCR repertoire breadth are dependent on the technical limitations of the assays being used to repertoire breadth. One of the first techniques used to estimate the breadth of the TCR repertoire was to spectratype the CDR3 region of the TCR according to size by polyacrylamide gel electrophoresis. Because of the random generation of a large number of unique CDR3 regions, the spectratype of CDR3 fragment lengths will form a Gaussian-like distribution of CDR3 amplicons of each length [145]. Though a step forward toward identifying TCR diversity, this methodology has been made largely obsolete by advances in high-throughput DNA and RNA sequencing, also referred as next generation sequencing (NGS), which have allowed for significantly deeper sequencing than is possible using capillary-based technologies [146, 147]. However, a recent comparison of various DNA and RNA based TCRseq methods found that α chain sequencing is particularly susceptible to reproducibility concerns, and while introducing a unique molecular identifier (UMI) may be more accurate, they are also more susceptible to missing rare clonotypes than non UMI-based methods [148]. Moreover, even if the per base-error of the NGS systems is low (far less than 1% using an Illumina sequencer), rare errors at a single nucleotide position are non-negligible as TCR sequences differing by even a single nucleotide can codify for different clonotypes [149]. Thus, NGS analyses pose a risk of not accurately capturing the breadth in, particularly, rare clonotypes. In addition, most studies performing TCR sequencing use bulk sequencing to obtain the highest number possible of TCR transcript reads; however, these attempts at ‘exhaustive sequencing’ are limited in their sensitivity and accuracy [150, 151]. These approaches also depend on sequencing only α and β chains without pairing, which considerably restricts the potential breadth of the TCR repertoire [152]; incorrectly estimating the number of unique clonotypes as a nonnegligible portion of $\alpha\beta$ T cells express two α chains and a single β chain (estimates of which vary from 14% to as many as $\sim 35\%$ of T cells expressing two alpha chains) [138, 153]. Although bioinformatic tools to reconstruct paired TCR alpha and beta chains have been proposed [154, 155], and these approaches have been useful to identify pathogen or antigen specific TCR clones [156, 157], as well as shared binding sites [158]; they are not particularly useful for estimating absolute repertoire breadth.

One approach to overcome many of the aforementioned limitations in estimating TCR repertoire breadth is to use single cell RNA sequencing (scRNAseq) technology. scRNAseq allows for a true assessment of TCR clonotypes by providing alpha and beta chain sequence pairing, which is not possible when performing bulk analysis. Importantly, scRNAseq also allows for analyzing the whole transcriptome of a single T cell identified through its TCR, which has been used great effect in studying the transcriptomic features of T

cells infiltrating tumors, such as in liver cancer [159]. However, while extraordinarily promising for overcoming the limitations of conventional bulk sequencing approaches and providing a true assessment of TCR repertoire breadth, the current limits of scRNAseq are the prohibitive cost.

Why does TCR diversity matter?

As discussed above, thymic involution, caused by either acute insult or chronic age-related decline, profoundly influences the peripheral T cell pool, primarily by tuning TCR repertoire breadth [144]. These shifts in repertoire likely underpin multiple clinical complications; in particular success of vaccination and of cancer immunotherapy, and possibly even disease incidence itself [67].

Traditional vaccination strategies rely on the presence of vast antigen recognition repertoires [41, 160]. Therefore, together with rapid viral modifications such as antigenic drift (a key feature of seasonal influenza virus), and constriction of the TCR repertoire with age as a result of declining thymic function; vaccination against influenza provides inadequate protection in elderly and immunocompromised individuals [161]. This can be specifically quantified as there is a demonstrably restricted influenza A specific V α and V β TCR repertoire with age [162]. Due in part to this tight correlation between thymic function and clinical outcomes, loss of naïve CD4+ T cells accompanied by a reduced TCR diversity has been proposed as a prognostic tool for determining responses to infection, especially in aging populations [55]. Furthermore, assessing infection or vaccine antigen-specific TCR diversity can be used as a surrogate readout of immune protection, as evidenced in studies using immunization against the alpha herpes family virus, varicella zoster [163] and hepatitis B infection [48]. Notably, the emergence of T cell directed vaccines, which induce both CD4+ and CD8+ effector responses, have proven to increase effective viral clearance [164], and although this is promising in immunocompetent individuals, the success is determined by a large pool of naïve T cells, further strengthening the need for therapeutic enhancement of T cell reconstitution for successful immunization.

Immunotherapy using antibodies directed against T cell checkpoint molecules has emerged as an enormously promising therapy for multiple malignancies [165, 166]. However, even in the best-case scenario, using dual blockade of both CTLA-4 and PD-1/PDL-1 in melanoma, this therapy leads to stable remission in only approximately 65% of recipients [167], and in cases such as lung cancer the success rate is significantly lower [168]. Recent work has revealed that some of the success of checkpoint blockade rests on the mutational burden of the tumor [169, 170]. That is, tumors with a higher rate of mutation (and subsequent neoantigens) have a greater chance of T cell receptor (TCR) recognition and more

successful outcomes from immunotherapy treatment. Given that T cells are central to antitumor immunity [171, 172] and that a diverse TCR repertoire promotes the probability of antigen recognition, immune response, and attenuation of disease progression [173, 174], the success of checkpoint inhibition, even in tumors with extensive mutations, is entirely predicated on the presence of reactive T cell clones against (most often unknown) tumor antigens. Therefore, constricted TCR repertoire breadth, regardless of the reason, is linked with poor response to vaccines and immunotherapy [175, 176], and TCR repertoire profiling can be used as a predictive biomarker for treatment success [177–179]. However, there are conflicting reports of the correlation of checkpoint blockade effectiveness with high mutational burden [180].

Thus, the combination of tumor genetics and the variability of tumor neoantigens, in addition to the breadth of the TCR repertoire, likely provides the most robust predictive insight into the efficacy of checkpoint inhibitor therapy. Therefore, enhancing TCR repertoire breadth could be of enormous clinical interest, both in the cases of cancer therapeutics, immunization, and endogenous response to infectious disease.

Repairing the thymus in settings of acute and chronic injury

Given the dynamic nature of thymic function and its importance for generating and maintaining an effective TCR repertoire, there is a clear clinical need for therapies that can boost thymic function. To this end, several putative therapies are being developed in preclinical models, some of which have gone on for assessment in clinical trials (Table 1; Figs. 1 and 2).

Cytokines and growth factors

One of the most widely studied molecules with thymic regenerative capacity is the lymphopoietic cytokine IL-7 [205], which can act directly on T and B lymphoid precursors [206, 207]. The mechanism behind IL-7-induced thymic regeneration lies in its ability to enhance the proliferation of lymphocytes and lymphoid precursors [208, 209]. IL-7 has been shown to be effective at boosting thymic function in both aged mice (chronic damage) as well as those receiving conditioning required for HCT (acute damage) [210, 211]. Since its discovery as a key lymphopoietic factor, IL-7 has been proposed as a therapeutic target for immune modulation [205]. Consistent with preclinical studies, clinical trials using recombinant IL-7 in patients with either solid tumors or HIV infection have shown that recombinant IL-7 is safe and led to expansion of both CD4+ and CD8+ T cells [191–195]. In the context of acute damage, recipients of allo-HCT who had been given a recombinant and glycosylated form of IL-7, CYT107, demonstrated a rapid increase in peripheral CD4+

Table 1 Therapeutic strategies to boost thymic function: preclinical development and clinical translation

	Preclinical		Clinical	Trial number	Refs
	Acute	Chronic			
Pre-T/HSPCs	++	ND	ND	N/A	[181–184]
RANKL	++	ND	ND	N/A	[81]
BMP4	++	ND	ND	N/A	[92–95]
IL-21	++	++	ND	N/A	[185, 186]
IL-12	++	++	ND	N/A	[187, 188]
ATO	++	ND	No data in regeneration but ATO have been used to determine thymic intrinsic defects in immunodeficient patients	N/A	[189, 190]
IL-7	++	++	Increased T cells in HIV+ patients and improved reconstitution in allo-HCT recipients	NCT03941769	[191–196]
KGF	++	++	Used widely for mucositis, trials have not shown considerable benefit in T cell reconstitution in recipients of HCT.	NCT01233921, NCT03042585, NCT02356159, NCT00593554	[77, 88, 95, 96, 98, 197–199]
IL-22	++	ND	Secondary readout in ongoing trial in steroid-refractory GVHD	NCT02406651	[33, 71, 81, 200]
Thymosin- α 1	++	++	Enhanced lymphocyte count and improved mortality in COVID-19 patients	NCT04320238 NCT04487444	[201, 202]
SSI	++	++	Increased thymic function and output of RTEs in prostate cancer patients (aged) and in HCT recipients	NCT01746849 NCT01338987	[203]
GH	++	++	Increased thymic size and RTEs	NCT04375657	[204]

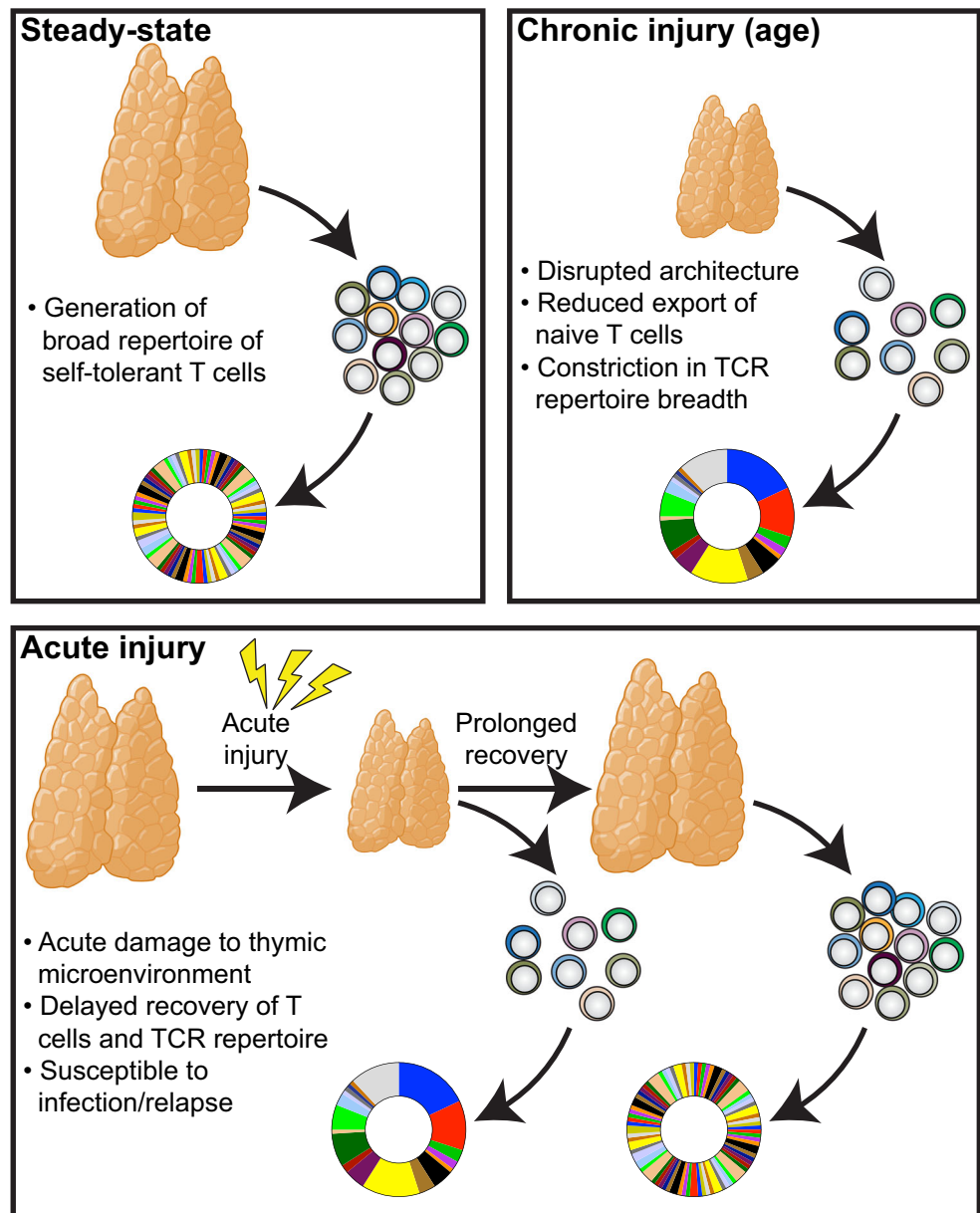
and CD8+ T cells and increased generation of virus-specific T cells [196]. However, exogenous IL-7 has significant effects on peripheral T cells and its effects on T cell reconstitution and repertoire diversity may primarily be by stimulating peripheral T cells, including RTEs [209, 212]. Currently, a clinical trial to evaluate the effect of recombinant IL-7 on T cell reconstitution in recipients of cord blood HCT is ongoing (NCT03941769).

One approach used to identify therapeutic strategies to boost thymic function has been to exploit the mechanisms that govern endogenous regeneration from acute injury [16]. As described above, studies have found that IL-22, KGF, RANKL, and BMP4 are all involved in the endogenous response to injury, and all can be utilized to boost thymic function in the setting of acute damage [77, 88, 95, 96]. Of these, the most widely studied has been KGF, with exogenous administration of recombinant KGF found to significantly increase thymic cellularity in mouse models of aging and following acute damage caused by radiation or chemotherapy [96, 98, 197]. Due to its approved status as a treatment for mucositis [213], KGF (palifermin; trade name Kevivance, marketed by Biovitrum) has emerged as a prominent potential therapeutic strategy for improving thymic function after acute injury such as in recipients of HCT. Several studies in mice and non-human primates demonstrate the efficacy of KGF for improving thymic-dependent T cell development in HCT [214]. However, in a recent trial of MS patients who have undergone a T cell depleting course of antiCD52 antibody, there was actually a detrimental effect of KGF on thymic-dependent T cell development. [198]. Several trials have been

initiated to explore the effects of KGF on T cell reconstitution in the setting of HCT (NCT01233921, NCT03042585, NCT02356159, and NCT00593554).

Although understudied clinically, BMP4, IL-22, and RANKL all show promise in their capacity to boost thymic function, at least in settings of acute injury. Due to the diverse pathophysiological roles of IL-22, and the key role in epithelial cell regeneration, modulation of the IL-22-IL22R system is an attractive therapeutic target. Several studies have found that exogenous administration of IL-22 could significantly improve thymic function after acute damage in mice [71, 81, 200]; including in the face of fulminant GVHD [33]. Furthermore, a recent clinical study suggests that serum levels of IL-22 could be predictive as an indicator of thymic output after HCT [215]. IL-22 production is triggered after damage by innate lymphoid cells, which also produce RANKL [71], which may regulate expression of IL-22 in an autocrine fashion [216], but also has its own regenerative capacity. In fact, administration of exogenous RANKL enhanced thymic function after bone marrow transplantation by boosting TEC subsets, including TEC progenitor niches [81]. While BMP4 itself could not be given to enhance thymopoiesis, a cellular therapy of thymic-derived BMP4-producing ECs could enhance thymic regeneration when given to mice after an acute form of damage caused by sublethal TBI but has not been tested in the chronic damage setting [95]. In addition to these factors, IL-21 has been shown in preclinical mouse models to be effective at reversing age-related involution and boosting thymic function after acute injury [185, 186]; and IL-12, which can induce IL-7, is capable of reversing age-related

Fig. 1 Damage and regeneration in the thymus and TCR repertoire. Steady-state T cell development in the thymus of young, healthy individuals results in the generation of a broad but self-tolerant T cell receptor (TCR) repertoire. It is this diversity of the TCR repertoire that allows for T cell recognition of unknown antigens. However, thymic function progressively declines from puberty resulting in reduced export of newly generated naïve T cells that, coupled with the expansion of existing naïve and memory T cells, leads to a constriction in the diversity of the peripheral TCR repertoire and reduced responsiveness to new antigens. Furthermore, even though the thymus has a remarkable capacity for repair after acute insults, and there is likely continual thymic involution and regeneration in response to stress and infection in otherwise healthy people, acute and profound thymic damage such as that caused by common cancer cytoreductive therapies or the conditioning regimens required for HCT, leads to prolonged T cell deficiency. In the setting of HCT, a well-established therapy with curative potential for a variety of malignant and nonmalignant diseases, delayed T cell reconstitution is an important contributor to transplant-related morbidity and mortality due to infections and malignant relapse. Elements of the figure were generated using [Biorender.com](https://www.biorender.com)



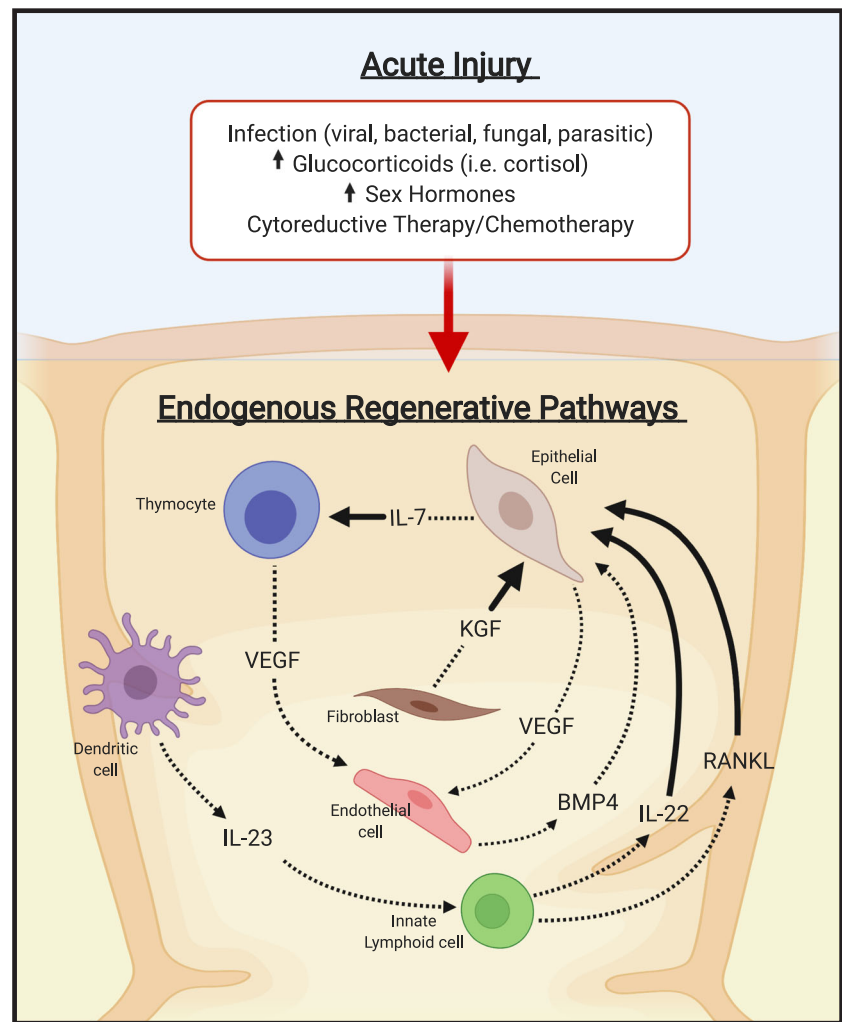
involution in mice [187]. Notably, IL-12 is also able to enhance engraftment of hematopoietic progenitors after irradiation [188].

Hormone modulation

Sex steroids, and especially testosterone, have been implicated in the age-related degeneration in thymopoiesis, B lymphopoiesis, as well as early lymphoid precursors [5]. Given these profound effects, perhaps unsurprisingly sex steroid inhibition (SSI) has been used to boost thymic function. SSI promotes reorganization of the thymic architecture, an enhanced ability to import circulating progenitors [217] and subsequently enhances

thymopoiesis in aged mice and humans [20, 60, 72, 217–219]. SSI has been shown to [1] promote lymphoid potential and overall function of hematopoietic stem and progenitor cells [20, 220, 221]; [2] induce the expression of CCL25 [217], which promotes the importation of hematopoietic progenitors from the circulation [222, 223]; and [3] induces the expression of the Notch ligand DLL4 [61]. SSI effects are not restricted to the thymus with significant effects in BM lymphopoiesis and on the earliest hematopoietic stem cells observed [63, 220, 221]. Furthermore, in addition to its impact on the aging thymus, SSI is also capable of significantly improving recovery following autologous [224] and allogeneic [225] HSCT as well as cytoreductive therapy [20, 72, 220]. SSI

Fig. 2 Acute injury and endogenous mechanisms of regeneration. (Top) Acute thymus injurious events including acute infection, rises in glucocorticoids and sex hormones, and cytoreductive treatment for HCT or chemotherapy for cancer treatment. (Bottom) Endogenous cell signaling pathways leading to regeneration from acute injury: IL-23 from thymus dendritic cells stimulate the release of IL-22 from innate lymphoid cells (ILCs). ILCs also release RANKL independent of IL-23. IL-22, RANKL, BMP4 (from thymic endothelial cells), and KGF (from thymic fibroblasts) act on thymic epithelial cells (TECs) stimulating their proliferation. TECs release IL-7 required for thymocyte repopulation. TECs release IL-7 required for thymocyte repopulation. Thymocytes produce Vascular Endothelial growth factor which signals to thymic endothelial cells. Bolded arrows indicate endogenous factors that, when given exogenously, have regenerative potential. Elements of the figure were generated using [Biorender.com](https://www.biorender.com)



can be achieved pharmacologically by disrupting upstream hormone signals, blocking the binding of sex steroid receptors, or by inactivating the hormones themselves [61]. Given that SSI is routinely used in prostate cancer patients, SSI is by far the most widely used therapeutic strategy with potential for boosting thymic function. In fact, SSI has clear clinical efficacy in both settings of acute insult and chronic age-related involution. Specifically, in a retrospective study of prostate cancer patients, there were increases in CD4 and CD8 counts as well as in TRECs [218]. In a follow-up prospective study, Boyd and colleagues found increased neutrophil engraftment as well as enhanced levels of TRECs and improved TCR diversity in recipients of both allogeneic or autologous HCT [203]. Two further prospective trials are currently recruiting to evaluate the effects of Luteinizing hormone-releasing hormone (LHRH) modulation for improving thymic reconstitution after allo-HCT using either the LHRH agonist leuprolide (Leuprorelin, NCT01746849) or the LHRH antagonist degarelix (Firmagon, NCT01338987).

In addition to sex hormone modulation, targeting growth hormone (GH, somatropin) and ghrelin have also emerged as promising strategies for thymic regeneration [226]. GH in particular is particularly promising given the extensive clinical studies evaluating its efficacy in a range of conditions. GH regenerates the aged thymus [227, 228] and enhances hematopoietic progenitor cell function in the BM [229]. As such, considerable interest in the translation of GH into the clinic has been shown, primarily in the setting of chronic HIV infection with enhanced thymus function and antiviral responses [230–233]. Although growth hormone has never been specifically studied clinically in the context of acute injury, a recent trial assessing chronological aging found that GH (in addition to the antidiabetic drugs metformin and DHEA) could enhance the number of RTEs and increased thymus size by MRI [204]. A follow-up expanded trial has been designed to assess current TRIIM-X trial (NCT04375657). Notably, a recent study found that thymosin- α 1, which has previously shown efficacy for T cell reconstitution in recipients of HCT [201], could also restore lymphocyte count and ameliorate mortality in COVID-19 patients [202].

Cellular therapies and artificial tissue

Given the systemic injury that occurs after acute injury caused by cytoreductive conditioning, including in the bone marrow, there is a profound lack in the supply of hematopoietic progenitors that are capable of seeding and reconstituting thymic function [234]. Therefore, one approach to boost T cell reconstitution after injury is to cotransplant BM-derived lymphoid precursors [181]. Although this approach is limited by the supply of lymphoid precursors that can be isolated from BM, ex vivo systems using Notch-1 stimulation may allow for the development of large numbers of T-lineage precursors that could be used for adoptive therapy [182, 183, 235–237]. Transfer of T cell precursors in a model of allo-HCT significantly enhanced thymopoiesis and enhanced peripheral T cell reconstitution [182–184].

All of the approaches discussed so far rely on the presence of functional endogenous thymus tissue, which may be lacking in particularly older individuals. Therefore one approach that has shown some promise is to build what has been called an artificial thymic organoid (ATO) entirely for regenerating immunity [238–241]. ATO tissues have been made by decellularizing endogenous thymic tissue, as well as generating synthetic matrices to support T cell development, though in vivo evidence of therapeutic efficacy of these approaches is still limited [189, 241, 242]. However, given the extensive requirement for cellular microenvironmental support for T cell development, these approaches still require cellular input to generate a fully functional thymus, namely the thymic epithelial microenvironment would need to be recapitulated. Thymic epithelial progenitor cells (TEPCs) have been successfully isolated from fetal mouse thymus and induced to generate a new thymus in athymic mouse recipients [243–246], and neonatal TECs, or TECs derived from pluripotent progenitors can promote enhanced thymic function [247–249]. However, while there is evidence of a bipotent TEPC in the postnatal thymus [250–252], their capacity to self-organize as a whole organ like fetal TEPCs is limited. TEC-like progenitors appropriate for this purpose have also been generated by direct conversion of embryonic fibroblasts by induced expression of the TEC transcription factor FOXP1 [253], as well as inducing TECs from embryonic stem cells or iPS cells from both mouse and human [92–94, 254]. Although the efficacy of ATOs have not yet been extensively evaluated in the setting of regeneration outside of mice, ATOs have recently been used to great effect to distinguish hematopoietic from intrinsic thymic defects in pediatric immunodeficiencies; thereby identifying patients with DGS that would be candidates for thymus transplant [190].

Conclusion

T cell immunity is critical for not only coordinating the adaptive response against pathogens but also for mounting a

response against malignancies. However, although the importance of the thymus for generation of an effective TCR repertoire is unquestionable, and there is a clear clinical need for boosting thymic function after immune depleting therapies such as the conditioning required for hematopoietic stem cell transplant (HCT); the importance of postnatal thymic function for clinical outcomes in a broader cohort of cancer patients is only beginning to be appreciated. In particular, wider use of new technologies such as single cell sequencing in particular will allow true evaluation of the breadth of the TCR repertoire and how this relates to pathophysiology of disease and therapeutics. Finally, new strategies are under development to enhance posttransplant T cell recovery and several of those are now in clinical trial, such as IL-7, KGF, IL-22, and SSI.

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