Thymic regeneration: teaching an old immune system new tricks

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Recent studies in mice and humans show that the importance of the thymus extends well beyond the initial seeding of the peripheral T-cell pool. Although peripheral homeostasis can maintain T-cell numbers, the thymus is the major, if not the exclusive, source of new T-cell specificities. With age, thymus atrophy dramatically reduces the export of new T cells and predisposes an individual to impaired T-cell function, reduced T-cell immunity, and increased autoimmunity. Thymus atrophy is also the primary obstacle to restoration of the T-cell pool in the aftermath of HIV treatment or lymphoablative therapies. Here, we review thymus T-cell production, with particular attention to the factors that influence thymocyte export, and examine the impact that recent thymic emigrants have on the peripheral pool. In the future, thymic regeneration might become a feasible and potentially powerful approach to rejuvenating a depleted peripheral T-cell pool.

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T cells are produced in the unique microenvironment of the thymus, from blood-borne progenitors derived from the bone marrow and fetal liver. Immature thymocytes accumulate beneath the thymus capsule, and then move inwards through the cortex and cortico–medullary junction (CMJ) toward the thymic medulla. They progressively encounter a variety of specialized stromal cells and undergo the multiple rounds of proliferation and differentiation that comprise the T-cell development pathway.

Mature, self-tolerant, major-histocompatibility-complex-restricted (MHC-restricted) T cells are exported to the periphery to seed, maintain or rebuild the different compartments of the peripheral T-cell pool, depending on circumstance. Several comprehensive reviews describe different aspects of T-cell development, including T-cell receptor (TCR) gene rearrangement [1], β selection [2], positive selection [3] and negative selection [4]. However, less well described are the events that immediately precede export from the thymus and the impact new T cells continue to have on the composition of the peripheral pool throughout life.

Export from the thymus

After a differentiation process of about four weeks (in mice), mature thymocytes congregate near the lymphatics and blood vessels of the medulla and are exported at a remarkably consistent rate of 1–2% of total thymocytes per day [5]. The emigration process is not well defined, but recent thymic emigrants (RTE) display little of the phenotypic or functional immaturity evident among most medullary thymocytes, suggesting that functional maturity probably determines when export from the thymus occurs [6].

The trafficking of thymocytes is mediated in part by the differential expression of chemokines and chemokine receptors on the surface of stromal cells and thymocytes, respectively [7]. Two chemokine–receptor pairs appear to be likely participants in the export process. Mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP) thymocytes express high levels of CCR7, which binds to the chemokine ligands CCL21 (SLC) and CCL19 (ELC) [8]. These ligands might foster emigration by attracting mature cells to the endothelium of blood vessels in the CMJ and medullary areas of the thymus, where expression levels of CCL21 and CCL19 are particularly high [7,9]. Targeted disruption of the CCR7 gene, or antibody-mediated blocking of the CCR7–CCL19 interaction, causes an abnormal accumulation of thymocytes and a significant fall in peripheral T-cell levels, consistent with export failure [9].

Another possible participant in the emigration process is the CCL25 chemokine. CCL25 is widely expressed by thymic stromal cells and its interaction with CCR9 plays an important role in the trafficking of $CCR9^+CD4^+CD8^+$ thymocytes through the cortex and CMJ of the thymus [10]. In mice, the downregulation of CCR9 by mature thymocytes might reduce their responsiveness to CCL25 and facilitate export by freeing cells to emigrate to the peripheral pool [8]. By contrast, in humans, medullary $CD8^+$ SP cells appear to remain responsive to CCL25 [7], and the interaction between CCL25 and CCR9 might promote the emigration of these thymocytes [11].

The variable expression of CCR7 and CCR9 by SP thymocytes is probably important for delivering mature cells to the areas where export occurs. However, their broad expression contrasts with the selective export of only the most mature cells. This suggests that delivering SP thymocytes to the appropriate area is only the first stage in a regulated process designed to prevent the release of immature cells to the periphery. Consistent with the idea that additional signaling is required, disabling G_i, protein pathways prevents thymic export and causes mature thymocytes to accumulate in the medulla, possibly through the inhibition of active repulsion signaling [12].

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Overexpression of the early-activation marker, CD69, has a similar inhibitory effect [13], as do various immunosuppressive agents, including FTY720 [14] and the imidazole-based compound THI [15], although their mechanisms of action are not well understood. Interestingly, a significant proportion of RTE divide immediately before export, in response to a TCR-mediated signal that is presumably triggered by self peptides presented in the context of MHC molecules [16]. This process might select thymocytes for export based on their acquired capacity to respond to TCR signaling and would be consistent with the ongoing, yet changing role of TCR-mediated signaling during thymic selection events and in the continual signaling required for the survival of T cells in the periphery [17].

Incorporation of recent thymic emigrants by the peripheral pool

In mice, RTE begin to seed the peripheral T-cell pool late in embryogenesis and continue to be exported from the thymus at a rate of 1–2% of thymocytes per day throughout life [5]. Because the size of the peripheral T-cell pool is thought to be homeostatically regulated, there was doubt about whether RTE are incorporated when T-cell numbers are normal and therefore, according to homeostatic theory, at a maximum [18]. However, contrary to suggestions that T-cell numbers are maintained at a maximum level, subsequent studies have shown that RTE are incorporated regardless of the pool's existing size [5,19]. This might be a reflection of the importance of RTE. Even when RTE numbers are dramatically elevated by transplantation of multiple thymuses, the peripheral pool continues to incorporate the additional T cells, rather than exclude them or delete pre-existing peripheral cells to maintain equilibrium. In mice, for example, the grafting of four additional thymuses caused the T-cell pool to grow and remain 80% larger than normal [5]. This is indicative both of the elasticity of the peripheral lymphoid compartments and of the potential availability of 'niches' to accommodate increased export levels. Interestingly, increases in the size of the T-cell pool occurred even with one additional thymus. In this instance, the daily increase in T-cell export represents <1% of the total T-cell pool, making it extremely unlikely that the additional RTE were overwhelming a predetermined homeostatic 'cap' on T-cell numbers. Instead, thymic export was having a measurable, direct impact on the size and composition of the T-cell pool.

Benefits of thymic export

RTE leave the thymus with their specificity for antigen determined by the productive rearrangement and expression of T-cell receptor genes. This random process occurs early in thymocyte development and the number of potential gene configurations far exceeds the number of T cells produced in a lifetime. Therefore, the accumulation of RTE provides for extraordinary receptor diversity and the pool is likely to contain T cells capable of recognizing and responding to virtually any pathogen the host encounters. By contrast, a pool built or maintained in the absence of thymic export (i.e. through homeostatic expansion of residual or transplanted T cells) gradually loses diversity, through the natural attrition of naïve cells and the expansion of the memory compartment. Although the overall number of T cells remains relatively normal, the increasingly homogenous T-cell pool has a reduced capacity to respond to new antigens, and imbalances between different subpopulations frequently occur.

The progressive loss of thymic function might be an important contributor to the immune dysfunction that occurs with age [20]. T cells continue to be exported from an aged thymus at an equivalent rate to a young one (~1% of thymocytes per day), but atrophy dramatically reduces its overall size and the number of RTE. In mice, atrophy begins from the time of sexual maturity [21]. Similarly, in humans, a dramatic reduction in thymus cellularity is seen in early adulthood, but whether the process is most pronounced from puberty, or occurs progressively from the postnatal period, is disputed [22,23]. Regardless of when atrophy begins, RTE export by an aged thymus can drop to fewer than 5% of that produced by a young adult. Although the thymus remains functionally competent, the export rate is insufficient to replace the naïve T cells lost daily from the periphery, and as the naïve pool shrinks, homeostatic proliferation is triggered and the memory pool expands [24] (Fig. 1). With time, these changes become more pronounced and are strongly associated with an increased incidence of autoimmunity, a reduced capacity for immune surveillance, and measurable declines in T-cell function at the level of individual T cells and within the pool as a whole [20,25]. The broader ramifications of these events are illustrated in several studies of elderly patients that demonstrate a strong correlation between the relative severity of age-related immune changes and lowered life expectancy [26–28].

It is not only with increasing age that an over-reliance on peripheral expansion, over thymic output, causes adverse changes in the composition of the T-cell pool. The phenomenon is typical of immune recovery following chemo- or radio-therapy, where 'space' created in the T-cell pool provides a strong stimulus to 'refill' the pool through proliferation. In these cases, the T-cell deficiency (lymphopenia) is usually so pronounced that homeostatic expansion occurs even in the presence of substantial thymic function. Although the proliferative restoration of cell numbers does improve immunity, full immune recovery is dependent on high thymic output of new RTE to replenish the naïve pool [29,30].

In adults in particular, recovery from lymphopenia usually involves a slow and partial restoration of the size, effectiveness and subset distribution of the T-cell

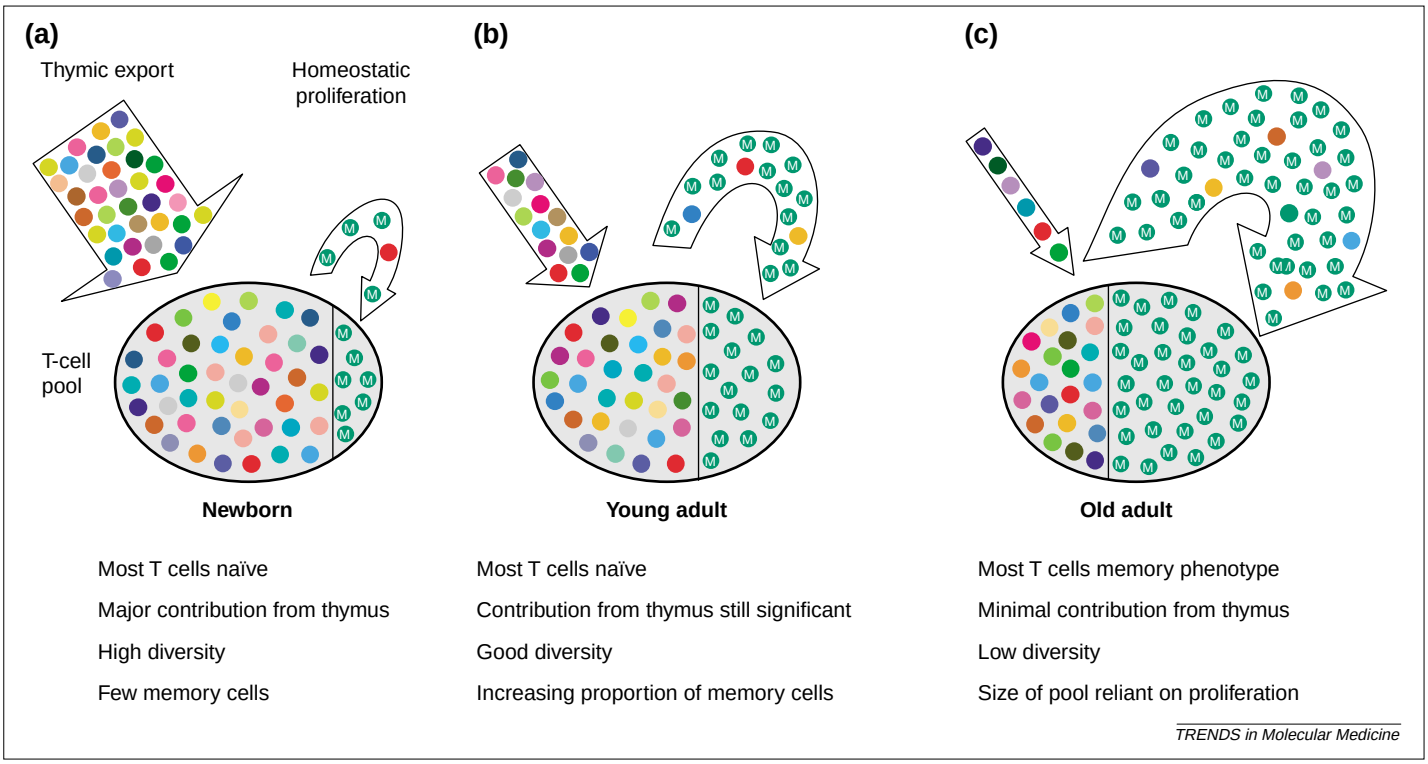

Fig. 1. Maintaining the peripheral T-cell pool with age. The size of the peripheral T-cell pool remains relatively stable over time, but T-cell diversity diminishes with age. In a young individual (a), the thymus exports sufficient numbers of naïve T cells (colored circles) to maintain the broad T-cell receptor diversity of the pool. As the thymus atrophies with age [(b), (c)], the number of thymic emigrants decreases and homeostatic proliferation of residual T cells is required to maintain the size of the pool. The consequence is a progressive increase in memory T-cell numbers ('M') and a subsequent loss of pool diversity.

population. The result is usually a relative paucity of naïve T cells and an over-representation of memory T cells [29,30] (Fig. 2). Hence, the reconstituted T-cell pool functionally and phenotypically resembles that associated with older age. In both cases, the diversity of the repopulated T-cell pool, and the distribution of T-cell subsets within it, are dramatically different from a normal young animal and can result in a measurable decline in immunocompetence. That these differences are greatest, and recovery is slowest, when atrophy of the thymus is most severe, illustrates the importance of thymic output in T-cell pool reconstitution and maintenance.

Homeostatic regulation of thymic export?

Given the impact of thymic export on the peripheral T-cell pool, it would be of great benefit if the active control of thymocyte export extended beyond a simple discrimination between mature and immature cells. For example, the size, composition and diversity of the peripheral T-cell compartment could theoretically be regulated by slowing thymic export when the periphery was 'full' and increasing it in times of lymphopenia [29]. A recent study described changed levels of T-cell receptor excision circles (TREC) in the periphery following hemopoietic stem cell transplantation, consistent with a homeostatically driven increase in thymic export [31]. However, changes in TREC concentration are an unpredictable guide to the rate of thymic output following myeloablative therapy because of the enormous changes the thymus and peripheral T-cell pool undergo during their reconstitution. Furthermore, the relatively small store of mature T cells in the medulla, and the considerable lag time taken to produce new T cells, suggest that the thymus is ill-equipped to respond rapidly to changes in the peripheral T-cell pool.

Using an experimental model to allow direct measurement of thymic export, we have confirmed that the thymus fails to vary the export rate or composition of the emigrants in response to changes in the composition or size of the peripheral T-cell pool [19,32]. Regardless of peripheral circumstance, the rate of emigration remained a product of thymus mass, with 1–2% of total thymocytes exported daily. This applied not only in normal mice, but also in instances where the size of the T-cell pool was increased by 80%, or depleted to <30% of normal. Furthermore, the composition of T cells exported was also unaffected by circumstances in the periphery; despite tight regulation of the $CD4^+$ and $CD8^+$ T-cell pool sizes [33], approximately two CD4 naïve T cells were exported alongside each CD8 cell (broadly reflecting the ratio seen normally in the thymic medulla and peripheral pool), regardless of induced changes to the peripheral CD4:CD8 ratio [32]. Taken together, these experiments show that the periphery has no detectable homeostatic impact on the export process.

RTE phenotype and detection

An evaluation of thymus function is particularly important for patients recuperating from lymphoablative treatments such as chemo- and radio-therapy, or from diseases that cause immunodepletion, such as HIV. Improved thymic function generally improves the rate, and long-term effectiveness, of T-cell reconstitution and immune recovery and, as a result, there is significant clinical

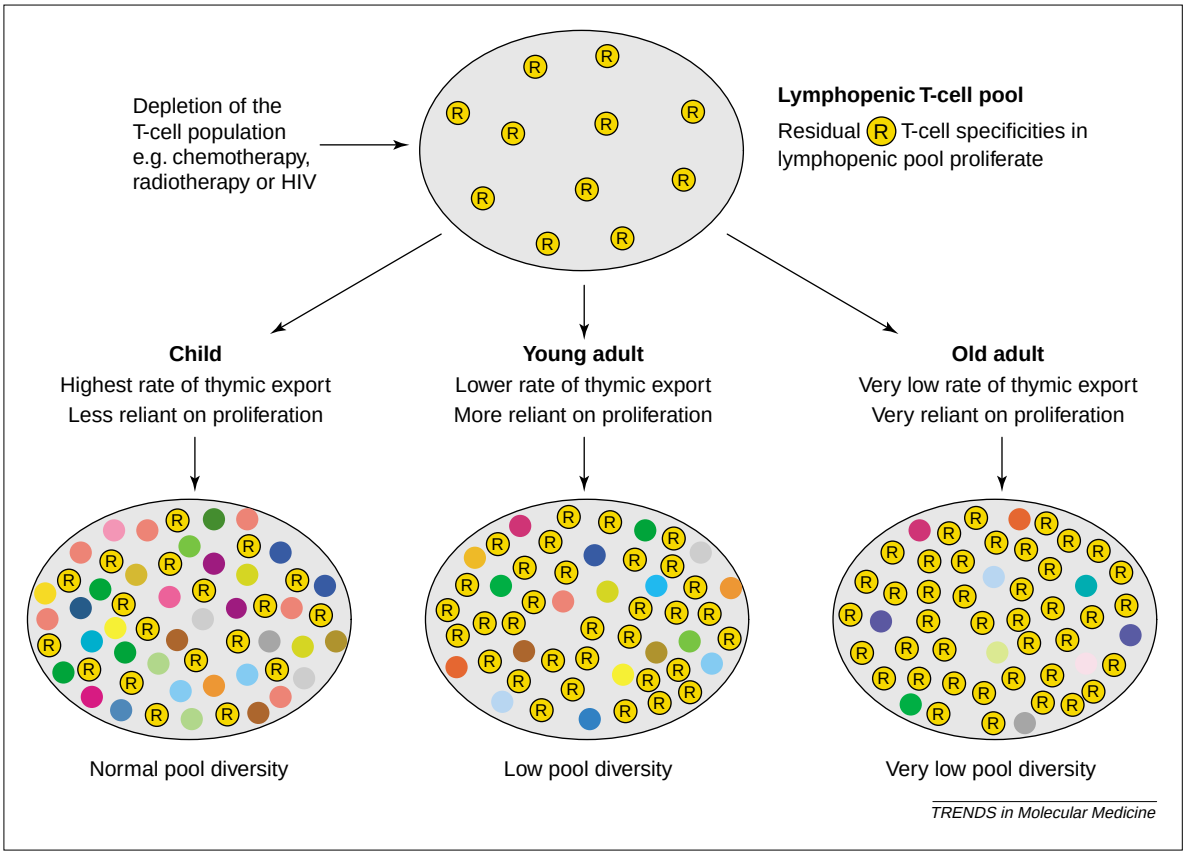

Fig. 2. Rebuilding the lymphopenic peripheral T-cell pool. The thymus has an important role in T-cell pool reconstitution. A depleted T-cell pool is typically restored through the export of naïve T cells from the thymus, and by the proliferation of residual peripheral T cells. Ideally, a balance will be reached between the diversity provided by new thymic emigrants, and the rapid recovery of T-cell numbers provided by proliferation. In the absence of sufficient thymic output, the size of the pool will still reach relatively normal levels, but the diversity will remain at levels reminiscent of the pool in its depleted state.

value in establishing a reliable system to measure thymic export levels [24,29]. Unfortunately, the task is difficult because it relies on identifying RTE among all the other cells of the T-cell pool. Accurate tracking of RTE in laboratory animals can be achieved using intrathymic injection of fluorescein isothiocyanate (FITC) [34]. In this protocol, all thymocytes are labeled randomly, enabling RTE in the periphery to be identified via flow cytometry, thus yielding an assessment of phenotype and rate of export. The technique has been successfully applied to a variety of animals models, but the requirement that dye must be injected intrathymically means that alternatives have had to be found for the study of human thymic export.

Attempts to distinguish phenotypically RTE from longer-lived naïve T cells, without thymic labeling, have been relatively unsuccessful. This is not entirely surprising given that RTE are generally regarded as members of the naïve T-cell pool; they express the low levels of CD44 and the high levels of CD62L characteristic of naïve T cells and, although many have divided immediately before export, like other naïve cells they are usually non-dividing except in circumstances of lymphopenia [35]. Some broad differences exist between RTE and longer-lived naïve T cells, at the population level, in the expression of Qa2, CD24, CTLA-4 and TSA-2 (mice), CD45RA (sheep), CD45RC and RT6 (rat) and CD103, CD31 and CCR9 (humans), but with the exception of the chT1 thymocyte antigen in chickens, no single marker has been identified that is uniquely expressed by either population.

T-cell receptor gene excision circles

Recently, many clinicians and researchers have come to regard the concentration of TRECs in the blood as a reliable indicator of RTE levels, and have extrapolated estimates of the rate of thymic export from these data [36,37]. TRECs are formed when developing thymocytes rearrange T-cell receptor genes, causing the excision of unused DNA fragments between the V, D and J elements [38]. The resulting TRECs are relatively stable, do not replicate during cell division, and are diluted between daughter cells following proliferation [39]. Therefore, newly exported RTE have relatively high TREC levels compared with T cells that have undergone one or more rounds of peripheral division.

Isolating the thymic contribution to changes in peripheral TREC levels is difficult because the PCR-based analysis measures the average TREC concentration within a large heterogeneous population (albeit often enriched for target cells), rather than individual cells. TRECs are present in all T-cell subsets at different levels, so changes in TREC concentration can theoretically result from changes in the proliferation, survival or distribution of any T-cell subset, not only RTE [39,40]. An additional complication is that thymocytes undergo multiple rounds of division, after the formation of TRECs, but before export [16,41]. The extent of proliferation

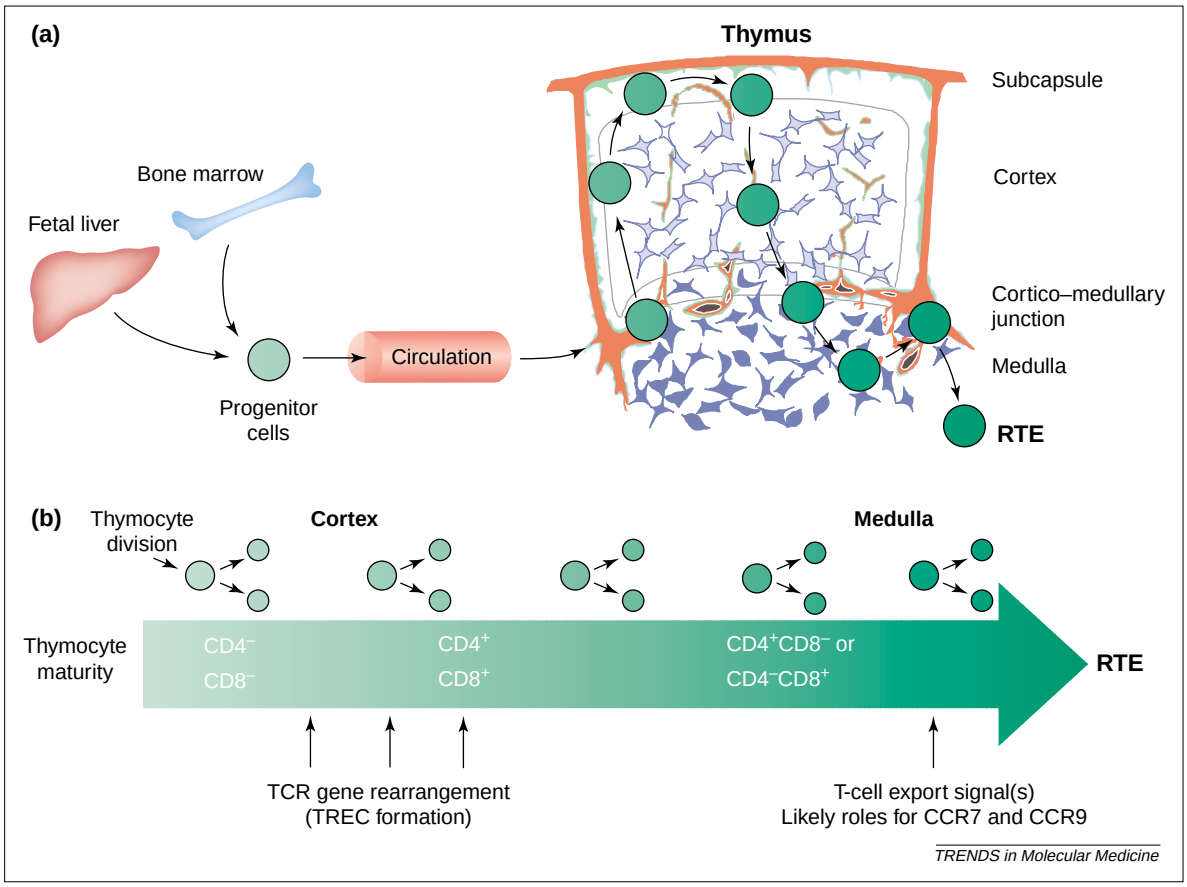

Fig. 3. Generating recent thymic emigrants (RTE). (a) Mature T cells of thymic origin are derived from progenitor cells produced in the bone marrow and fetal liver. After entering the thymic microenvironment, immature thymocytes traffic through a variety of stromal cell types and undergo a complex series of proliferative and differentiation events that results in the production of mature T cells. (b) Thymocyte trafficking and export are thought to rely heavily on interactions between chemokines produced by the thymic stroma and chemokine receptors (CCRs) expressed by thymocytes. Thymocyte export is often assessed by measuring the levels of T-cell receptor excision circles (TRECs) in the blood. TRECs are formed early in thymocyte development when T-cell receptor gene rearrangement takes place. However, it is important to recognize that thymocytes proliferate after the formation of TRECs, resulting in the dilution of TREC levels within the cell, before export. The extent of division varies between cells and this can impact on the reliability of the assay as a measure of thymic function.

varies from cell to cell, so the TREC concentration is not uniform for all RTE, even at the time of export (Fig. 3). This makes it impossible to correlate TREC concentration closely with RTE numbers and particular caution must be exercised when interpreting TREC data from patients whose treatment regime might affect intrathymic proliferation. In rodents, the potential for misinterpretation can be reduced by also measuring the TREC concentration among thymocytes [42], but this is unlikely to be possible in clinical settings.

In the periphery, changes within the naïve, activated or memory T-cell pools can affect TREC levels independently of thymic output. RTE and long-lived naïve T cells contain similar amounts of TRECs, as demonstrated in studies where TRECs were readily detected decades after thymectomy [43]. Ideally, long-lived naïve T cells would be identified and excluded from analysis but RTE and naïve T cells cannot be easily separated. Fortunately, most instances of significant T-cell proliferation and death occur within the pool of activated and memory T cells, which has comparatively low TREC levels. However, these changes still affect the frequency of TRECs within the overall pool and can lead to an incorrect assessment of altered thymic function [40]. It is possible to reduce the potential for misinterpretation by measuring TREC levels at multiple time points so that only changes occurring within the time window between assays affect the comparison.

Furthermore, by assessing the expression of proliferation (e.g. Ki67), naïve or memory (e.g. CD44, CD45RA/RO), apoptotic (e.g. annexin V) and activation (e.g. CD25, CD69) markers, at each time point, it should be possible to take into account most non-thymic influences [40,42].

It remains problematic that measurement of thymic output is often most valuable in immunodepleted patients, in whom extensive homeostatic T-cell division is likely to be occurring. However, with careful consideration of the aforementioned caveats, TRECs still represent the best means of quantifying thymic export in humans. The majority of analysis is concerned with identifying a possible increase in thymic output and given that non-thymic factors generally reduce TREC concentration, the worst-case scenario is usually an underestimate of thymic emigration. An overestimate of thymic export could theoretically result from TRECs generated by secondary TCR gene rearrangement during selection processes in the thymus, or in the periphery after export, but the incidence of such events is relatively low and is unlikely to impact analysis [44,45].

The future: therapeutic reversal of thymus atrophy

Many immune problems associated with aging or lymphopenia could, in theory, be alleviated by increased thymic export. The plasticity of the T-cell pool makes it receptive to this form of intervention, but because the rate of thymic export (when measured

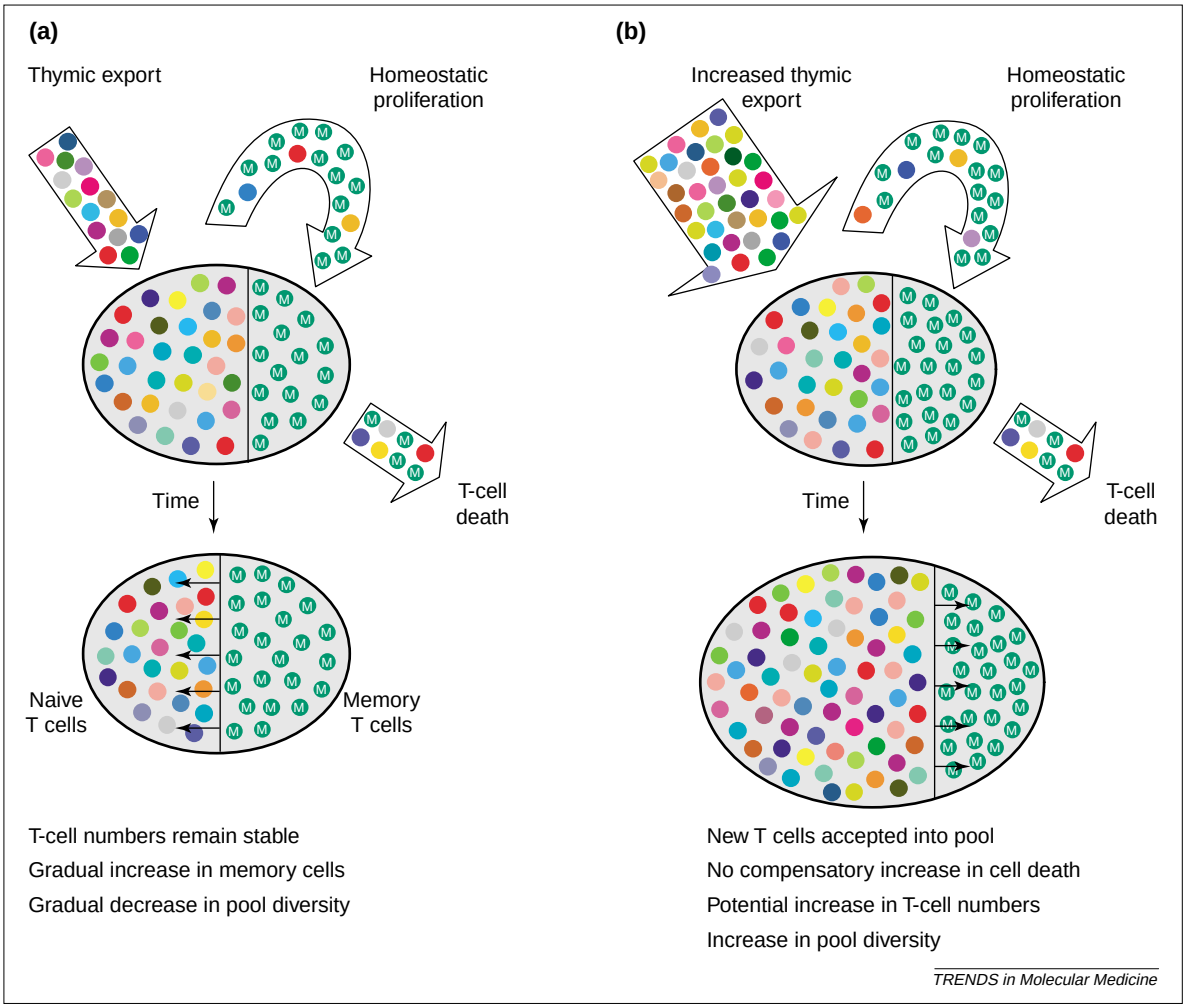

Fig. 4. Correcting an imbalanced T-cell pool with increased thymic export. (a) Naïve and memory T cells each fill important and mutually exclusive roles in maintaining effective immunity, so maintaining a balance is crucial. However, disproportionately high numbers of memory cells can accumulate if thymic export levels are low, or if considerable homeostatic proliferation has taken place. (b) Increasing the rate of thymic export can correct the imbalance by restoring the optimal number and proportion of naïve T cells. If sufficiently large, an increase in the rate of thymic export can result in an overall increase in T-cell pool size.

as a proportion of thymus size) is not responsive to imbalances in the peripheral pool, the only means of producing a sustainable increase in export levels is to increase thymic mass (Fig. 4).

In mice, the transplantion of additional thymuses is an established means of manipulating the composition of the T-cell pool, and in humans, pieces of postnatal thymus have been transplanted to treat genetic disorders such as DiGeorge syndrome (a disease where the thymus is virtually absent) [46]. Despite problems such as donor tissue availability and the requirement for significant surgery, this makes thymic transplantation a realistic option for restoring the size and distribution of T-cell subsets in the recuperative period following chemo- or radio-therapy, or in the wake of infectious diseases such as HIV.

One way to reduce the problems associated with transplanting an intact thymus could be to transplant thymic progenitor cells, capable of differentiating and growing within the host. Our laboratory [47], and that of Blackburn *et al.* [48], recently identified multipotential, epithelial precursor cells within the embryonic mouse thymus that, when transplanted, grew to form an architecturally normal, and fully functional thymus. Similar cells remain to be identified in humans, but the possibility of culturing and storing such cells *in vitro*, and the probability of less invasive surgery, make this a promising avenue of research.

Several alternatives to thymus transplantation are also emerging, including *in vitro* systems of *de novo* T-cell differentiation for transplantation of new T cells grown from $CD34^+$ progenitor cells. In one of these studies, a matrix of biocompatible metal seeded with thymic stromal cells and hemopoietic stem cells produced a functional, 'synthetic' thymus from which mature T cells emerged after two weeks of culture [49]. Other *in vitro* systems use cultured thymic tissue to support T-cell development, but their complexity, variability and low yields make them unlikely candidates for therapeutic applications. The matrix technology is in its infancy, but providing the limitations can be overcome, this type of system might eventually produce sufficient T cells *in vitro* (and possibly *in vivo*) to benefit patients.

For many patients, the preferred alternative would be to increase the rate of export from their own atrophied thymus without surgery. A more complete understanding of the thymic export process might eventually identify factors (e.g. chemokines) capable of inducing short term increases in the emigration

rate, but sustainable improvements are likely to require the reversal of thymus atrophy. One proven strategy is to lower the circulating levels of sex steroids. In aged rodents, inhibition of sex steroid function by chemical or physical castration leads to long-term recovery of thymic cellularity and function [50–52]. For humans, long-term chemical castration (sex-steroid ablation therapy, based on elevated levels of luteinizing-hormone-releasing hormone (LHRH) agonists) is regularly used in the treatment of breast cancer, endometriosis and prostate cancer. This might also be a powerful approach for the therapeutic reversal of thymic atrophy.

One variation could be the temporary use of steroid antagonists to maximize thymic export during the most crucial periods of pool recovery, possibly in conjunction with cytokine treatment. For example, even though levels of IL-7 remain stable with age, IL-7 treatment stimulates growth within the thymic stromal compartment and promotes T-cell development by increasing the early stages of thymocyte differentiation [52]. On its own, treatment with IL-7 is least effective in the aged and has the side effect of promoting homeostatic expansion among peripheral T cells [42,53]. However, when administered alongside LHRH agonists, IL-7 could optimize the immediate recovery of T-cell numbers through proliferation of residual T cells, while also prompting the longer-term recovery of pool diversity, by restoring thymic function. Growth hormones and growth-hormone secretagogues are also reported to increase thymic mass [21], and the inhibition of thymosuppressive cytokines, such as IL-6, leukemia inhibitory factor, macrophage-colony stimulating factor, stem cell factor and oncostatin M, might have similar effects [24]. Targeting these factors could prove useful in future efforts to rejuvenate the thymus.

It is perhaps surprising that more effort has not been made toward prolonging or restoring thymic function in humans after puberty. This might be because age-related deterioration of the immune system is often not immediately apparent to the patient, nor is it typically debilitating. Although testament to the ability of the T-cell pool to maintain effective, albeit suboptimal, immunity for extended periods of time, this reduces the immediate medical requirement (and incentive) to reverse, or prevent, atrophy. We are presently investigating the different factors involved in human and murine thymic atrophy. In mice, the aged thymus can be successfully regenerated, and thymic export dramatically increased, by manipulating steroid pathways. The challenge will be to provide the benefits of thymic regeneration to aged and immunodepleted patients while minimizing the side effects of currently available therapies.

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Hepatitis C: molecular virology and antiviral targets

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Chronic hepatitis C is a leading cause of liver cirrhosis and hepatocellular carcinoma worldwide. Although current treatment options are limited, progress in understanding the molecular virology of hepatitis C has led to the identification of novel antiviral targets. Moreover, *in vitro* and *in vivo* model systems have been developed that allow systematic evaluation of new therapeutic strategies. This review details current concepts in molecular virology and emerging therapies for hepatitis C.

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Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) [1]. It is estimated that 170 million people worldwide are infected with HCV. The seroprevalence rate is ~1% in Western Europe and North America. With the introduction of anti-HCV screening of blood and blood products in 1990, new cases of post-transfusion hepatitis C have virtually disappeared and intravenous drug use has become the major identifiable mode of transmission in many countries. Acute hepatitis C is usually asymptomatic and results in chronic infection in 50–80% of cases. Approximately 20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years. Once cirrhosis is established, the rate of HCC development is 1–5% per year. The mechanisms underlying viral persistence and pathogenesis are poorly understood [2]. A protective vaccine does not currently exist and therapeutic options remain limited. At present, pegylated interferon-α (IFN-α) in combination with ribavirin is the treatment of choice for chronic hepatitis C. However, only ~40% of patients infected with HCV genotype 1 achieve a sustained response [3]. Moreover, in clinical practice many patients do not qualify for IFN-α therapy because they have contraindications or ongoing substance or alcohol abuse. Hence, overall, only a minority of patients with chronic hepatitis C can be successfully treated [4]. As a consequence, projections indicate that the mortality rate from HCC associated with chronic hepatitis C will further increase for the next 15–20 years [5]. Thus, there is an urgent need to develop more effective and well-tolerated therapies for chronic hepatitis C.

HCV was identified more than a decade ago by the use of recombinant DNA technology [6,7], but investigation of the replication cycle has been limited by the low viral titers found in sera and livers of infected individuals and the lack of an efficient cell culture system or small animal model permissive for HCV. Nevertheless, considerable progress has been made using heterologous expression systems, functional cDNA clones [8] and, more recently, subgenomic replicons [9] (see Refs [10,11] for reviews).

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