Neonatal Thymectomy in Children - Accelerating the Immunological Clock?

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Abstract

The thymus is critical for central tolerance and diverse T-lymphocyte repertoire development, to provide lifelong defence against pathogens, whilst maintaining self-tolerance. Peak thymic output occurs in-utero, infancy and early childhood, diminishing throughout life. Infants with congenital heart disease requiring sternotomy, often undergo thymectomy to clear the surgical field. Longterm effects of early thymectomy are just being appreciated. Many patients remain asymptomatic, despite immunological findings mirroring those of immunosenescence. Few develop increased infection or lympho-reticular malignancy risk.

When considering effects of infant thymectomy, patients with partial DiGeorge syndrome or hypomorphic *RAG* mutations may be instructive. These patients are lymphocytopenic, with increased early onset infection and autoimmunity risk, not seen in most infant thymectomy patients. Thymic structure of partial DiGeorge syndrome or hypomorphic *RAG* patients is abnormal, with disrupted architecture inclining to perturbation of central tolerance. Similar findings may be seen in patients with myasthenia gravis, although disrupted peripheral tolerance may play a greater role in autoimmunity development.

In conclusion, infant thymectomy may increase future risk of infection or autoimmunity with premature immunosenescence, mediated through disruption of central and peripheral tolerance mechanisms, initiated by early cessation or diminution of thymic output. Ideally, some thymic tissue should be preserved at time of surgery.

Keywords

Thymus, neonatal thymectomy, immunosenescence, T-lymphocyte, immunodeficiency.

Abbreviations

Acetylcholine receptors (AChR), anti-nuclear antibodies (ANA), anti-neutrophil cytoplasmic antibodies (ANCA), Cytomegalovirus (CMV), congenital heart disease (CHD), Epstein Barr virus (EBV), Myasthenia Gravis (MG), post-transplant lymphoproliferative disease (PTLD), regulatory T-lymphocytes (Tregs), thymic (natural) Tregs (nTregs), peripheral (induced) Tregs (iTregs), T-cell receptor excision circles (TRECs), T-cell receptor (TCR),

Introduction

The thymus is the primary lymphoid organ supporting the development of T-lymphocytes capable of reacting with harmful foreign antigens but recognizing and tolerating harmless self-antigens. Complete or partial thymectomy performed during congenital cardiac surgery in infants and young children procedures, improves surgical access and allows visualization and cannulation of great vessels[1]. However, the partial or complete removal of the thymus in early life may have significant health impacts in adulthood, although the data describing this are mixed.

Several studies have described T-lymphophenotype changes and early immunosenescence occurring after early thymectomy for cardiac surgery, although findings differ between reports, most of which are limited to small patient cohorts, usually with no appropriate controls. The short-term clinical impact of early thymectomy does not result in severe infectious complications, but long-term consequences are poorly defined. In other conditions where post-thymectomy long-term follow-up data are available, e.g. myasthenia gravis (MG), significant late-onset autoimmune and lymphoproliferative complications are reported[2,3]. Similar complications may occur in early thymectomized patients following cardiac surgery[4]. We describe current understanding of clinical and immunological consequences of infant or early childhood thymectomy during cardiac surgery, and explore the link with immunosenescence.

Normal thymic structure and function

The thymus is critical for T-lymphocyte development, providing an optimal microenvironment in which developing thymocytes undergo proliferation, T-lymphocyte receptor rearrangement, and differentiation into mature T-lymphocytes,

which are released into the circulation. Mature T-lymphocytes generate effective responses against pathogens and tumor antigens, but are tolerant of self and harmless environmental antigens[1,5-9]. A critical thymic function is generation of central tolerance occurring in two phases.

- positive selection in the thymic cortex, with apoptosis of T-lymphocytes that fail to recognize self-MHC molecules

- negative selection in the thymic medulla[6], resulting in the removal of high affinity self-antigen reactive T-lymphocytes that harbor the potential to elicit autoimmunity.

Negative selection is facilitated through central expression of a wide range of peripheral tissue-restricted self-antigens by medullary thymic epithelial cells, partly controlled by the Autoimmune Regulator (AIRE) transcription factor[6] and Fezf2[10].

A proportion of T-lymphocytes with high affinity to self-antigen survive negative selection and develop into regulatory T-lymphocytes (Tregs), related to anti-apoptotic signals associated with FOXP3 expression. FOXP3 is expressed mainly by $CD4^+CD8^-$ T-lymphocytes, but expression has been observed in double positive $CD4^+CD8^+$ T-lymphocytes, so the point at which the fate of these cells is determined remains unclear. Approximately 80% of the Treg repertoire originates in the thymus[11], but they can be generated extra-thymically by differentiation in the periphery (inducible, iTregs). As the properties and origin of these Treg populations differ, discriminating between them is important to better understand their specific functions in regulating immune homeostasis. Markers to differentiate nTregs and iTregs include expression of the transcription factor Helios, a member of the *Ikaros* transcription factor family, and the surface antigen neuropilin-1[12], both apparently

highly expressed in nTregs. However, recent studies suggest that expression of these two markers does not unequivocally identify Treg clones of thymic or peripheral origin[13], and a specific lineage marker for Tregs remains to be identified.

Various methods quantify thymic export of naive T-lymphocytes including enumeration of T-lymphocyte receptor excision circles (TRECs) and specific cell surface markers. TRECs are stable circular episomal DNA segments generated as a by-product of TCR gene rearrangement, not duplicated during mitosis and thus diluted with cellular division[7,14]. Circulating TREC-containing naive T-lymphocytes generation can persist for many months after thymectomy[15,16] and extra-thymic TRECs may further complicate interpretation. Despite this, PCR-quantification provides an accepted and practical measurement of thymic output by calculating the frequency of TRECs in a defined population of cells or absolute number of TRECs per millilitre of blood[15]. Cell surface markers CD45RA, CD27 and CD62L have also been used to identify naive T-lymphocyte populations, although expansion can occur without loss of these markers. $CD31^+CD4^+CD45RA^+$ T-lymphocytes have a high TREC content declining with age, reflecting decline in thymic function[15,17]. $CD45RA^+CD27^+$ and $CD45RA^+CD31^+$ T-lymphocytes correlate strongly with TRECs in pediatric patients, suggesting either panel can be used for enumerating peripheral blood naïve T-lymphocytes[9,15,17,18].

Another indicator of normal thymopoiesis is a broadly diverse TCR repertoire, necessary to respond adequately to a wide range of antigens[9]. Quantification of TCRVβ family T-lymphocyte expression is a useful tool to detect repertoire

restriction, indicating limited T-lymphocyte pool diversity associated with reduced thymic output[7,9].

Normal age-related thymic involution and immunosenescence

The thymus reaches maximum volume and output during the first year of life, followed by a rapid decline in thymic function between 1-8 years, proceeded by a gradual decline in function with increasing age[14,19]. Age-related thymic decline is a chronic irreversible process[19], attributed to a reduction in the number of developing intrathymic T-lymphocytes and alterations to the thymic stromal microenvironment, particularly with loss of thymic medullary epithelial cells and replacement by connective and adipose tissue.

Age-related thymic atrophy consequences include decreased peripheral naive T-lymphocyte export and TCR repertoire contraction[6]. The decline in thymopoeisis, and life-long chronic antigenic stimulation, result in peripheral memory T-lymphocyte predominance, with preservation of immune responses to ‘known antigens’ but possible failure to respond to new antigens[20]. Recent evidence suggests the existence of heterogenous populations of memory T-lymphocytes. In mice, central memory $CD4^+$ T lymphocytes contain subpopulations with different degrees of turnover. The maintenance of $CD4^+$ T-lymphocyte memory subsets relies heavily on this recurrent replenishment of new cells sourced from the naive pool. If these observations are also relevant in humans, early thymectomy is likely to have a significant negative impact on replenishing the $CD4^+$ T-lymphocyte memory pool[21]. Accumulation of memory T-lymphocytes accounts for the shift from Th1 to Th2 cytokine profiles, promoting humoral immunity, production of autoantibodies and

potential development autoimmunity. Other immunological changes reported include reduced T-lymphocyte proliferation due to a decline in IL-2 and IL-2 receptors[22]. nTreg production is not impaired with age-related thymus atrophy as decreased negative selection due to thymic atrophy is compensated by maintained, or increased, nTreg production[23].

Progressive thymic function decline, alterations to the peripheral T-lymphocyte compartment and decline in T-lymphocyte function, as well as a shift in cytokine profiles, is attributed to the 'immune risk phenotype'[24], responsible for increased susceptibility to infection, cancer and autoimmunity occuring with advancing age[25]. Elderly patients are predominantly at risk of cancers derived from tumor neoantigens, whereas immune responses to shared tumor antigens are well-retained[20]. Although an increase in autoantibodies is observed in the elderly, this does not always translate into increased autoimmunity[25] demonstrating that precise mechanisms linking altered ageing immune responses with development of autoimmune disease remain unknown[24]. Some studies suggest contraction of the TCR repertoire may be not be as dramatic[20,26], and the continued, albeit reduced, thymic export of naive T-lymphocytes into the peripheral pool continuing into old age provides sufficient supply to maintain immunological protection[27,28] suggesting involvement of other factors in the clinical consequences of immunosenescence.

Post infant thymectomy immunological and clinical consequences

As long-term follow-up data become available, the immunological impact of early pediatric thymectomy becomes appreciable (Table 1). Gradual $CD4^+$, but also $CD8^+$, T-lymphocytopenia develops[1,7,31]. Progressive decline in naïve T-lymphocytes and

TRECs is observed, with an increased proportion of peripheral memory T-lymphocytes, mirroring the immunosenescent phenotype previously described[1,7.9]. These changes are reversible if thymic regeneration occurs[31-33], emphasizing the importance of sparing some thymic tissue at neonatal thymectomy. The frequency of Ki67-expressing naive T-lymphocytes increases in thymectomized patients compared to age-matched healthy controls, reflecting increased peripheral replication in response to thymic output decline[18]. Most studies suggest a steady gradual decline in cellular-mediated immunity following thymectomy. A seminal study looking at young adults, aged 18-26 years who had been thymectomized in the first two weeks of life for surgery on transposition of the great arteries. Study subjects had $CD4^+$ and $CD8^+$ lymphocytopenia with a loss of naive cells, reduction in diversity and increase in oligoclonal memory cells, with a switch to a pro-inflammatory cytokine profile. A subgroup of these patients had an altered T-lymphocyte profile normally related to cytomegalovirus (CMV) infection leading to induction of strong CMV-specific T-lymphocyte responses. In the absence of adequate T-lymphocyte renewal from the thymus, these responses may deplete the naive T-lymphocyte pool. This is a pattern seen in elderly individuals, and indicative of an immune risk phenotype, which is predictive of increased mortality[34].

An 18 year follow-up study of thymectomized patients demonstrated quantitative naive T-lymphocyte decline, TCR repertoire contraction and skewing reflecting a reduced naive T-lymphocyte peripheral pool with expansion of pre-existing T-lymphocyte clones, and potentially reduced T-lymphocyte proliferative ability indicated by shorter telomere length[1]. However, T-lymphocyte function, proliferative responses and IFN-γ production were normal[7]. Higher $CD8^+$ T-lymphocyte

expression of PD1 (expressed following T-lymphocyte activation and used as a marker of exhaustion) was demonstrated in patients who had previously undergone sternotomy to repair congenital heart disease (CHD) at <12 months age compared to similar age-matched patients without sternotomy. This may reflect T-lymphocyte dysfunction, although clinical significance is undetermined[9].

An increased proportion of Tregs within the $CD4^+$ T-lymphocyte pool has been observed post-thymectomy, possibly due to preferential Treg proliferation. A decline in $CD4^+CD25^+CD127^{low}CD45RA^+$ naïve Tregs and expansion in $CD4^+CD25^+CD127^{low}CD45RO^+$ memory Tregs with greater suppressing potential, has been observed, which may reflect compensatory peripheral tolerance secondary to lack of post-thymectomy central tolerance[1]. However, evidence from murine models suggests that in severe lymphopenia, $FOXP3^+$ nTregs can lose their suppressive capacity, and revert to T_H cells which promote tissue infiltration and damage[35]. It is not clear whether this has relevance in humans. One study found an increased percentage of $FOXP3^+CD4^+$ T-lymphocytes, but predominantly comprising $CD45RO^+FOXP3^{high}$ suppressive Tregs and $CD45RO^+FOXP3^{low}$ Tregs, shown to be non-suppressive[36]. These data suggest a potential risk factor for the development of autoimmunity in early thymectomized patients.

One cohort of 178 patients with single-ventricle CHD and early thymectomy demonstrated difficulty clearing molluscum contagiosum, and had a high incidence of human papillomata[37]. Generally, however, short-term immunological impacts post-thymectomy appear benign, as other studies with longer patient follow–up do not replicate these findings.

Absence of significant infections following neonatal thymectomy may be explained by the diverse naive T-lymphocyte reservoir present at birth, following normal fetal

thymic development and function, which exists prior to thymic ablation and age-related decline. This may protect against serious infections in the short- to medium-term. In contrast, in congenital thymic aplasia, such as complete DiGeorge syndrome, an absent or restricted oligoclonal naive T-lymphocyte population at birth predisposes early to significantly impaired adaptive immunity, presenting in infancy with severe, persistent and opportunistic infection.

Longer-term follow-up in cardiac thymectomy patients demonstrates early-onset immunosenescence, predisposing to later-onset complications including development of EBV-associated post-transplant lymphoproliferative disease (PTLD). A 14% incidence of PTLD was demonstrated in a pediatric cohort post-cardiac transplant, occurring in patients with CHD rather than cardiomyopathy[4], possibly reflecting the impact of early cardiac surgery in patients with CHD prior to transplant. In another study, almost all patients who developed PTLD underwent infant surgery, suggesting that early thymectomy resulted in dysregulated T-lymphocyte immunity and impaired EBV responses[38]. Given the evidence that early thymectomy gives rise to an altered CMV-related T-lymphocyte profile in some patients[34], it is possible that a similar disruption in anti-EBV T-lymphocyte immunity, possibly associated with a 'second hit' such as prolonged immunosuppression following cardiac transplantation, renders some patients more susceptible to PTLD.

Humoral changes likely reflect changes in T-lymphocyte homeostasis. Immunoglobulin disturbances after thymectomy include reduced IgA and IgG subclass levels:- alterations in B-lymphocyte subsets have not been studied[5,29]. One study examined pneumococcal polysaccharide antibody response in paediatric

patients who had received a cardiac transplant and were receiving long-term immunosuppression. Patients transplanted before 4 years of age were more likely when older to have impaired pneumococcal polysaccharide antibody responses, and low IgG2 antibody levels than those transplanted after 4 years of age[30]. B-lymphocyte numbers were normal and immunosuppression regimens similar in both groups, raising the possibility that altered T-lymphocyte help may be implicated.

The appearance of autoantibodies is described in patients with early thymectomy, with detection of ANA or ANCA antibodies in half of thymectomized patients in one cohort, increasing with age[29], although this is not replicated in all studies[5]. Patients with autoantibodies had more memory T-lymphocytes compared with those without autoantibodies, and with healthy controls. An increase in IgG autoantibodies, associated with autoimmune disease, and decrease in IgM natural autoantibodies, associated with maintenance of self-tolerance, was observed, possibly reflecting diminished maintenance of self-tolerance. These studies mirror the ageing immune system with thymic involution, suggesting early onset of immunosenescence.

Despite the presence of autoantibodies in thymectomized patients, clinical autoimmune disease is not frequent, possibly due to preferential proliferation of Tregs and an increase in their proportion after thymectomy, suppressing autoreactivity[29]. As autoantibodies can arise long before clinical symptoms develop however, further monitoring is necessary to determine whether clinical autoimmune disease develops later in life.

The partial DiGeorge syndrome model

DiGeorge syndrome, frequently caused by 22q11.2 deletion, is the most common, well-defined thymic primary immunodeficiency, with a spectrum of presentation

ranging from athymia to normal thymic function. Most patients have partial DiGeorge syndrome, associated with diminished but not absent thymic output. The immune changes described in these patients can be compared with those observed in thymectomized pediatric patients, but with consideration of the main difference between them: the presence of a healthy pool of naïve T-lymphocytes prior to surgery in thymectomized patients in contrast to diminished, restricted pool of naïve T-lymphocytes in DiGeorge syndrome.

Partial DiGeorge syndrome patients exhibit diminished $CD4^+$ and $CD8^+$ T-lymphocyte numbers, reduced naïve T-lymphocytes and TRECs, normal B-lymphocyte numbers but with increased naïve B-lymphocytes and perturbed memory B-lymphocytes[39-41]. TCR repertoire analysis demonstrates an increase in oligoclonal peaks and Vβ family dropouts compared to controls, indicating TCR repertoire restriction, similar to that described in thymectomized patients[42]. However, DiGeorge syndrome patients experience more infections and autoimmunity than reported in thymectomized patients. Of a large cohort of partial DiGeorge syndrome patients, 64% had recurrent and/or severe infections and 7.8% had autoimmune disease with onset from 7.8 years of age[8]. Dysphagia, anatomical palatal alterations and gastrointestinal reflux were identified as predisposing factors related to upper respiratory tract infections, whilst autoimmune disease was related to $CD4^+$ T-lymphocytopenia and increased memory $CD4^+$ T-lymphocytes[39]. Clinical or laboratory immune abnormalities and autoimmunity were related to reduced $CD3^+CD4^+CD45RA^+CD31^+$ T-lymphocyte recent thymic emigrants, supporting the role of deficient thymopoiesis in development of autoimmunity in these patients[39,43]. In one study, an increased risk of non-cardiac death, attributed to severe infection or

proliferation was associated with reduced naive $CD4^+$ T-lymphocytes and $CD8^+$ cytotoxic T-lymphocytes[44].

Recent data describing changes in thymic structure and function may help explain, at least, the increased risk of autoimmunity – thymii from patients with 22q11 deletion demonstrated hypoplasia, diminished maturation of the medulla, a reduction in the number of $AIRE^+$ medullary thymic epithelial cells, and a reduced number of thymocytes[45]. These findings suggest that there is likely also to be compromised thymic negative selection, impairing central tolerance and increasing the risk of autoimmunity.

These findings contrast with those of patients with “partial FOXN1 deficiency”, due to heterozygous mutations in *FOXN1*. Affected infants have low TRECs and T-lymphocytopenia, and some have experienced recurrent infections, including severe infections. Unlike patients with partial DiGeorge syndrome, autoimmunity does not appear to be a significant feature amongst infants or adults with heterozygous *FOXN1* deficiency[46]. This implies that T-lymphocytopenia alone does not predominantly drive autoimmunity, but impaired central tolerance is an important risk factor, as seen in partial DiGeorge syndrome[45] and also patients with hypomorphic mutations in *RAG1/2*, where T-lymphocytopenia is also associated with thymic dysfunction and impaired central and peripheral tolerance[47,48], as well as a decrease in T-lymphocyte receptor diversity[49,50].

DiGeorge syndrome patients may have an increased malignancy risk, including lymphoma, neuroblastoma, acute lymphoblastic leukemia, osteosarcoma, Wilms tumor, thyroid carcinoma, and hepatoblastoma, although whether this is due to deletion of certain genes (e.g. SMARCB1 or COMT), related to immunodeficiency, or

other mechanisms, remains unclear[51,52]. Management recommendations for these patients include immunological follow up and indications for antimicrobial prophylaxis, vaccination, and immunoglobulin replacement where appropriate[53,54]. Similar recommendations are lacking in “acquired” thymic deficiency.

The Myasthenia Gravis Model

Symptoms of MG are characterized by muscle weakness and fatigue, and are due to a decrease in acetylcholine receptors at neuromuscular junctions, secondary to autoantibodies directed against the acetylcholine receptors (AChR) in the majority of patients, or against neighboring proteins aiding the clustering of acetylcholine receptors. Around 75% of AChR Ab-positive patients with anti- AChR antibodies have thymic abnormalities, with germinal center formation or hyperplasia in approximately 65%, or thymoma in approximately 11%[55,56]. These thymic abnormalities facilitate the development of T-lymphocytes autoreactive to skeletal muscle proteins through presentation of cross-reactive epitopes by thymic epithelial cells[57,58]. Thymectomy is second line therapy for management of MG; mechanisms by which patients respond to thymectomy are unclear[59], although removal of the thymus may eliminate a source of continued antigen presentation, and thus reduction in T-lymphocyte-driven auto-reactive B-lymphocyte stimulation. Since MG mainly affects adults, comparing data from this patient group with a pediatric population is challenging due to differences of immunological maturity. However, longer follow-up data in the MG population are available compared to that of pediatric patients post-cardiac surgery, providing important insights.

Decreased T-lymphocyte counts, reduced TCR Vβ repertoire and TREC concentration, increased Tregs and normal B-lymphocyte counts, are observed during long-term follow up in MG thymectomized patients unlike normal controls and non-thymectomized MG patients[2,16,60], similar to that of thymectomized pediatric cardiac patients. Severe infections have not been reported in thymectomized MG patients[61].

There is a high incidence of autoimmune disease after thymectomy[59,62], with increased IgG, IgM, anti-cardiolipin and anti-dsDNA antibodies in MG-thymectomized patients compared with the other groups studied[2]. A recent study demonstrated a 6.25-fold increased incidence of autoimmune diseases (including rheumatoid arthritis, systemic lupus erythematosus and Sjogren's Syndrome) compared to healthy controls. In contrast, MG patients treated with plasmapheresis did not develop autoimmunity[3]. This may support a link between thymectomy and subsequent development of autoimmune disease. However, patients undergoing thymectomy are more likely to have thymic abnormalities including thymoma or germinal center hyperplasia, which may induce other autoimmunity in addition to MG, as well as having less severe disease. The use of plasmapheresis in severely affected patients may clear other autoantibodies and mask other autoimmunity. Homeostatic regulation of T-lymphocytopenia may also impact on clinical consequences. T-lymphocyte proliferation in a lymphocytopenic environment occurs to re-balance T-lymphocyte homeostasis, and try and maintain T-lymphocyte diversity and functionality. At least two forms of T-lymphocyte proliferation can be observed in murine models:- a rapid spontaneous IL-7 independent proliferation which leads to broad differentiation of memory cells and a slow homeostatic IL-7 dependent proliferation which results in more limited differentiation[63]. The

predominant method of proliferation in a particular clinical setting may influence the likely development of autoimmunity.

Less is known about the relationship between thymectomy for MG and the risk of developing malignancy; thymic and extrathymic tumors have been described before[64], concomitantly and after the diagnosis of MG. This makes it difficult to draw firm conclusions between the relationship between thymectomy for MG and subsequent malignancy risk, which is already increased with advancing age[65].

Conclusions

Most children with CHD survive into adulthood and it is therefore important to understand long-term consequences of early thymectomy. The immunological effects may not be apparent until several decades later, and mimic physiological immunosenescance, associated with increased infections, autoimmunity and malignancies, albeit occurring earlier than expected. The immunosenescent phenotype of thymectomized patients resembles that which occurs with normal ageing. In the short- and medium-term, infection and autoimmunity do not appear to be increased, but the longest published follow-up data available are 18 years after thymectomy. This population may be at future risk; further monitoring may determine if observed immunological changes translate to clinical problems later in life.

However, the clinical consequences may depend on factors additional to the original surgery, including thymic regeneration following partial thymectomy, intrinsic thymic dysfunction leading to perturbed central and peripheral tolerance and diminished T-lymphocyte receptor diversity (Figure 1), or slow homeostatic T-lymphocyte proliferation leading to reduced diversity. Additionally, in patients who subsequently undergo cardiac transplantation and longterm immunosuppression, immunological

consequences of early thymectomy may be enhanced, with poor polysaccharide antibody responses and increased risk of PTLD. Further studies may define if autoimmunity is also an increased risk, and determine the lifelong consequences of neonatal or infant thymectomy. In the meantime, efforts to preserve at least some thymic tissue during cardiac surgery should be advocated.

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Figure 1. Possible models for development of infection risk or autoimmunity. In patients subject to infant thymectomy, early complete disruption of thymopoiesis may lead to peripheral T-lymphocyte expansion, with alterations in natural and induced regulatory T-lymphocyte populations, contraction of repertoire and skewing to a memory phenotype, with failure to replenish the peripheral T-lymphocyte pool. In myasthenia gravis, onset is much later, leading to a normal peripheral T-lymphocyte pool until adulthood. Abnormal thymic architecture, possibly with alterations in peripheral tolerance may predispose to autoimmunity and infection post thymectomy. Disturbed central tolerance due to disrupted thymic medullary archictecture leads to early onset lymphocytopenia, restricted repertoire and premature onset autoimmunity in partial DiGeorge and hypomorphic RAG patients, whilst in healthy individual, late onset physiological failure of thymopoiesis precedes peripheral T-lymphocyte expansion, enlargement of the T-lymphocyte memory pool, and gradual acquisition of autoreactive T-lymphocytes through homeostatic proliferation.