

MATURATION AND MIGRATION OF MURINE CD4 SINGLE POSITIVE THYMOCYTES AND THYMIC EMIGRANTS

Xi Xu ^{a,*}, Qing Ge ^{a,*}

Abstract: T lymphopoiesis in the thymus was thought to be completed once they reach the single positive (SP) stage, when they are “fully mature” and wait to be exported at random or follow a “first in-first out” manner. Recently, accumulating evidence has revealed that newly generated SP thymocytes undergo further maturation in the thymic medulla before they follow a tightly regulated emigrating process to become recent thymic emigrants (RTEs). RTEs in the periphery then experience a post-thymic maturation and peripheral tolerance and eventually become licensed as mature naïve T cells. This review summarizes the recent progress in the late stage T cell development in and outside of the thymus. The regulation of this developmental process is also discussed.

REVIEW ARTICLE

Introduction

The thymus provides a unique microenvironment for the development and maturation of T lymphocytes. The key events during T cell development include: the entry of lymphoid progenitor cells from the bone marrow into the thymus and the differentiation into T cell precursors; the formation of functional T cell receptor (TCR) through TCR β - and α -chain rearrangement; positive and negative selection to ensure the major histocompatibility complex (MHC) restriction to self-peptide as well as clearance of autoreactive cells [1-4]. After a highly regulated developmental process in the thymus, only about 1% of the thymocytes are able to emigrate and join the peripheral lymphocyte pool [5,6].

Compared to the early stages of thymocyte development where detailed developmental process and the underlying mechanisms are reasonably well defined, the late stage development, in particular, the development of CD4 or CD8 single positive (SP) thymocytes, the thymic egress, and the post-thymic maturation of recent thymic emigrants (RTEs) have been largely ignored. SP thymocytes were thought to be “fully mature”, and could leave the thymus at random or follow an ordered “first in-first out” manner [7]. Recent studies, however, revealed a dynamic and eventful developmental program for SP thymocytes in the thymus as well as RTEs in the periphery. The migration of these young T cells from the thymus to the periphery was also found to be tightly regulated. As the peripheral maturation of RTEs has been discussed in detail by Fink et al. [8,9], we mainly summarize the recent progress on the maturation and emigration of SP thymocytes.

The development of SP thymocytes in the thymus

The migration and residence of SP thymocytes in the thymus

After positive selection, the survived CD4 and CD8 double positive (DP) thymocytes relocate from the thymic cortex to the medulla, down-regulate one of the coreceptors and become CD4 or CD8 SP thymocytes. A direct precursor-product relationship between dividing cortical DP cells and mature medullary SP thymocytes was estimated to be within 1-3 days using bromodeoxyuridine (BrdU) incorporation approach [10,11].

The residence time of SPs in the thymic medulla, however, varies from 4 to 12 days based on different experimental settings. For instance, with continuous [³H] thymidine incorporation, Egerton et al. demonstrated that the complete replacement of the medullary compartment took about 12 days [12]. The results from pulse labeling with BrdU suggested that the turnover of BrdU⁺ SP thymocytes occurred in 5-7 days [13]. Similarly, the intrathymic delivery of MHC-expressing adenoviruses into MHC class II-deficient mice led to a conclusion of 6-7-day of residence time [14]. The adoptive transfer of the earliest SP thymocyte subset also resulted in a 4-7-day persistence in the thymus before egress [15]. Despite the differences in suggested residency time, it remains to be determined how SP thymocytes are regulated to finish the maturation program and central tolerance before acquiring egress capability.

The developmental program of SP thymocytes

With the help of many newly discovered cell surface molecules, investigators came to realize the heterogeneity of medullary SP thymocytes. For instance, based on the expression of CD24 (HSA), SP thymocytes can be divided into two subgroups, with the CD24⁻ ones being more mature than the CD24⁺ ones, producing more cytokines upon activation [16]. CD69 is expressed in only a fraction of TCR⁺ SP thymocytes [17], whereas Qa2 is expressed in SPs with more mature functions [18]. Thus, several developmental pathways have been proposed to link the phenotypic differences of SPs with their functional maturity. Take a two-stage scheme for example, cells

^aKey Laboratory of Medical Immunology, Ministry of Health. Department of Immunology, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xue Yuan Road, Beijing 100191, P R China

* Corresponding author. Tel.: +86 1082802593; Fax: +86 1082801436
E-mail address: xuxi@bjmu.edu.cn (Xi Xu); qingge@bjmu.edu.cn (Qing Ge)

at the early stage with a phenotype of CD69⁺CD24⁺Qa2⁻, barely responded to Con A or anti-CD3 stimulation while those at the late stage with a phenotype of CD69⁻CD24⁺Qa2⁺ responded quite well by proliferation and secretion of a variety of cytokines [13,19-21]. A different combination of CD69 and CD62L expression revealed another two-stage scheme for SP thymocytes: CD69⁺CD62L^{lo} and CD69⁻CD62L^{hi} [22]. However, other evidence has suggested the existence of intermediate stages [23,24].

Based on the expression of CCR7, two types of three-stage models were proposed for the studies of negative selection and nTreg development. The first one demonstrated that CD4 SP thymocytes can be divided into SP1 (CD24⁺CCR7⁻), SP2 (CD24⁺CCR7⁺), and SP3 (CD24⁻CCR7⁺) [11]. The second one combined CD69, CCR9 with CCR7, and suggested a slightly different developmental program: CD69⁺CCR7^{-/lo}CCR9⁺, CD69⁺CCR7⁺CCR9⁻, and CD69⁻CCR7⁺CCR9⁻ [25]. However, no functional studies were undertaken to compare these subsets.

Recently, we have resolved TCRαβ⁺CD4⁺CD8⁻ thymocytes into four subsets: SP1 (6C10⁺CD69⁺), SP2 (6C10⁻CD69⁺), SP3 (CD69⁻Qa2⁻), and SP4 (CD69⁻Qa2⁺) (Figure 1) [26]. The functional comparison, microarray analysis, and the sequential appearance of these subsets during mouse ontogeny and after the intrathymic adoptive transfer of SP1 cells have confirmed that these four subsets define a sequential and irreversible multistage program for the development of CD4 SP thymocytes [15,26]. The cell proliferation and cytokine secretion upon Con A or anti-CD3 and anti-CD28 stimulation, such as IL-2, IL-4 and IFN-γ, were found to be progressively enhanced from SP1 to SP4 [15,26]. The migration from the thymic cortex to the medulla may not occur until SP thymocytes reach SP2 stage according to the different expression of PlexinD1 and CCR7 (PlexinD1 suppresses the signaling of CCR9/CCL25 for cortex retention and CCR7 promotes the cortex-to-medulla migration of thymocytes) in SP1 and SP2 subsets [27]. Based on the expression of S1P₁ and the phenotype of fluorescein isothiocyanate (FITC)⁺ T cells in the periphery 24 hours after FITC intrathymic injection, SP4 thymocytes were believed to be capable of emigrating from the thymus [27,28].

When investigating the developmental program of CD4 SP thymocytes in the thymus, one important issue is the interference of T cells that have recirculated back to the thymus from the periphery. Recirculating T cells in the thymus can be detected in mice from 2 days to nearly 2 years of age [29,30]. In young adult mice, only 1-2% of CD4 or CD8 single positive cells in the thymus are recirculating T cells. The proportion increases dramatically with age, reaching over 20% in mice nearly 2 years of age [29,30]. Recirculating T cells are phenotypically more mature and some express activation markers when compared to newly generated SP thymocytes [29]. The role of these cells on the maturation of SP thymocytes is not clear yet. However, these cells need to be excluded when analyzing SP thymocytes. A purification strategy of selecting CD44^{lo} SP thymocytes or GFP⁺ SP thymocytes (RAG-GFP transgenic mice) could help us exclude the returning CD44^{hi} T cells and GFP⁻ mature T cells in the thymus.

Negative selection and the thymic microenvironment

Negative selection is a process in which newly generated T cells are rendered non-reactive to self-antigens and those with strong reactivity to self-peptide-MHC complex are deleted by apoptosis. Studies in the last twenty years have revealed that negative selection mainly takes place in the thymic medulla [13,31,32]. Thus, defects in T cell migration towards the thymic medulla or mice with

disorganized thymic medulla are often associated with an impaired negative selection and increased autoreactive T cells in the periphery. Examples of these include mice deficient in *Ccr7* [33], *Relb* [34-36], *Nfkb2* [37], *Nik* [38], lymphotoxin β receptor (*Ltbr*) [39], *Traf6* [40], or autoimmune regulator (*Aire*) [26,41-43]. Among these molecules, CCR7 regulates the cortex-to-medulla migration of SP thymocytes, whereas the rest are all involved in thymic epithelial cell differentiation and function [33-43].

Medullary thymic epithelial cells (mTECs) and medullary dendritic cells (mDCs) are two main antigen presenting cells (APCs) in the thymus (Figure 1) [3,44]. mTECs express a variety of tissue specific antigens (TSA) normally found in the periphery. This is partially attributed to the function of Aire [42,45-48]. Indeed, AIRE deficiency leads to autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in human and similar organ-specific autoimmune diseases in mouse [42,49,50]. Increasing evidence suggests that the role of Aire is not limited to the regulation of TSA expression. Aire also affects antigen processing by mTECs as well as appropriate differentiation of mTECs [44]. NF-κB signaling plays an important role in regulating Aire expression and mTEC maturation. Thus, the perturbation of this signaling pathway, such as in mice deficient in LTβR, its ligands LTβ or Light, CD40, and Rank, results in defective negative selection and increased autoimmunity [39,41,51,52].

In addition to mTECs, mDCs are also very important for negative selection. Selective depletion of thymic DC in a transgenic mouse model resulted in an increased frequency of CD4 SP thymocytes and the development of autoimmunity [53]. The processing and presentation of TSA by mDCs is derived mostly from DCs' uptake of apoptotic mTECs [54-56]. Migratory DCs from the peripheral blood also bring peripheral antigens to the thymus, thus further promoting central tolerance [57-59].

Although it is now well accepted that negative selection takes place in the thymic medulla, it remains largely unknown at which developmental stage of SP thymocytes that negative selection starts and at which stage it ends. In *Relb*^{-/-} mice that have defects in negative selection due to the significant reduction of mTECs and mDCs, a developmental blockage was observed between SP3 and SP4 subsets. Such developmental arrest was also revealed in *Aire*^{-/-} mice. This suggests that the SP3/SP4 transition could be a critical checkpoint for CD4 SP thymocyte development and negative selection [26]. Interestingly, Cowan et al. reported that the development of Qa2⁺ SP thymocytes could be supported by *Relb*-deficient mTECs in the model in which a lymphoid fetal thymus organ culture from *Relb*^{-/-} embryos was grafted into wild type mice [25]. As the evidence from our group and Fink group suggested that DCs could promote the upregulation of Qa2 in SP thymocytes and RTEs [28,60], it is likely that wild type DCs in Cowan's model migrate to the grafted thymus and promote the phenotypic maturation of SP thymocytes. However, whether the functional maturation of CD4 SP thymocytes is impaired in this model is awaiting for further examination.

Recently, the expression of Ikaros family transcription factor Helios was used to mark the SP thymocytes that undergo negative selection. Daley et al. showed that with the coinduction of Helios and the proapoptotic protein Bim, CCR7⁺CD4⁺CD69⁺ thymocytes up-regulated PD-1, down-regulated CD4 and CD8 and underwent Bim-dependent apoptosis. On the contrary, Helios⁺CCR7⁺CD4⁺ thymocytes revealed Card11- and c-Rel-dependent activation that opposes Bim-mediated apoptosis. Such activation did not result in the proliferation of SPs due to the lack of growth mediators such as IL-2 and Myc [11]. However, the role of this "hollow" activation of

autoreactive cells and the eventual fate of these Helios⁺CCR7⁺ cells remain unclear.

Thymic output

The overview of thymic output

After highly regulated developmental process in the thymus, only about 1% of the thymocytes are able to emigrate as RTEs and join the population of peripheral lymphocytes. By comparing the phenotype of SP thymocytes and thymic emigrants within 24 hours of egress, Dong et al. demonstrated that the main thymic population in adult mice that enters the periphery is the functionally most mature SP4 subset with a phenotype of CD69⁻Qa2⁺. In neonatal mice, however, the RTE precursors bear a phenotype of SP3 cells (CD69⁻Qa2⁻) [28]. It awaits for further investigation whether such phenotypic difference of RTEs is due to a difference in progenitors in newborn versus adult mice or unique mechanisms to promote a fast establishment of the peripheral T cell pool by premature thymic egress [61]. Despite the difference in phenotypic composition, the export ratio of RTEs to total thymocytes keeps constant during lifetime in healthy individuals, while the absolute number changes with the size of the thymus [5,8,29,62]. Thus, the detection of RTEs in the periphery was often used as a marker to predict the status of thymopoiesis in patients with various diseases. Before the mechanism of egress is completely understood, it is hard, however, to use the numbers of circulating RTEs to distinguish between the impaired thymopoiesis and defective egress under certain disease states [63-65].

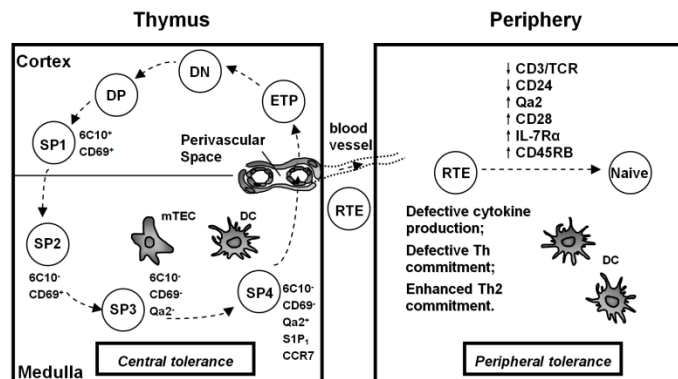


Figure 1. Late stage development of murine CD4 single positive T cells in and outside of the thymus. ETP, Early T lineage progenitor; DN, double negative; DP, double positive; SP, single positive; RTE, recent thymic emigrants; mTEC, medullary thymic epithelial cells; DC, dendritic cells.

The export of thymocytes to the periphery is thought to occur mainly through perivascular space (PVS) (Figure 1). The thymic PVS is composed of a vascular basement membrane and a second basement membrane bordering the thymic parenchyma [66]. While some studies suggested that the PVS was located in the medulla and cortico-medullary junction of the thymus, others claimed that PVS could also be found in the thymic cortex [67-70]. The presence of lymphocytes in normal PVS has been observed by transmission electron-microscope (TEM) and scanning electron-microscope (SEM), while a giant PVS filled with thymocytes could also be found in the non-obese diabetic (NOD) mice under fluorescence microscope [69-72]. The agonist of S1P, FTY720, an immunosuppressive compound that perturbs S1P- and S1P₁-mediated signals, inhibited the egress of thymocytes and caused the

accumulation of mature thymocytes in the PVS. It suggests that thymic PVS may be the main site for thymic output [33,73-75]. More direct evidence was revealed by intravenous injection of PE-conjugated CD4 antibody. Within 5 minutes of injection, CD4⁺ T cells in the PVS could be selectively labeled, with the great majority located within 50 μm of the cortico-medullary junction on thymic cross sections. Calculating the number of labeled cells per thymus further confirmed that the majority of thymocytes emigrate via PVS at the cortico-medullary junction [6]. Moreover, lymphatic vessels could also be involved in thymocyte emigration, though they might be more important in aged or diseased animals [76,77].

The regulation of thymocyte egress

The mechanisms controlling thymic output remain elusive until recently. Early studies suggested, based on relatively mature but heterogeneous phenotype of RTEs, that thymocytes leave the thymus at random or follow an ordered “first in-first out” manner [7]. Recent studies, however, revealed more complex regulation.

It was demonstrated that sphingosine-1-phosphate (S1P) and one of its receptors, S1P₁, can regulate thymocyte emigration. S1P₁ is a G protein coupled receptor (GPCR) that expressed in mature SP thymocytes, while S1P is produced by vascular endothelium as well as neural crest-derived pericytes that ensheath the blood vessels [6,78-80]. The high concentration of S1P in the blood and around PVS attracts S1P₁-expressing mature thymocytes to export [6,81]. The importance of S1P-S1P₁ signaling in regulating thymocyte emigration is further evidenced by mice deficient in S1P₁, sphingosine kinases (essential for the production of S1P) or lipid phosphate phosphatase 3 (LPP3, can degrade thymic S1P), with significantly decreased thymic output and accumulated thymocytes in the thymic medulla [6,79,80,82]. Based on these data, the search for thymic RTE precursors was much easier as the expression of *S1pr1* (the gene that encodes S1P₁) was found highest in SP4 thymocytes. The expression of *Foxo1*, the transcriptional factor that regulates the expression of *S1pr1*, also peaked at SP4 thymocytes, suggesting that CD4 SP thymocytes may not acquire the ability to egress until the cells reach SP4 stage [27]. Other factors, such as KLF2 (transcriptional factor of S1P₁), PI3K (negative regulator of KLF2) and PTEN (negative regulator of PI3K) can also influence thymocyte emigration [83-85].

In addition to S1P-mediated chemotaxis, CCR7, another member of the GPCR family, and one of its ligands CCL19, also contribute to thymocyte emigration. CCL19 attracts mature T cells migrating out of the fetal thymus organ culture through its interaction with CCR7. Interestingly, another ligand of CCR7, CCL21, fails to show the involvement in thymic emigration [86]. The chemorepellent signals provided by thymic stroma, including the chemokine stromal-derived factor (SDF)-1 (or CXCL12), which repels T cells via a CXCR4 receptor mediated manner, might also be important for thymic emigration [87,88].

Except for GPCR mediated thymic emigration, other factors such as early growth response gene 1 (Egr1) [89], aryl hydrocarbon receptor (AHR) [90], laminin-5 [91] and VLA-5 (integrin α5β1) [92] have all been demonstrated to be involved in the egress process. Egr1 is a transcriptional regulator whose expression can be induced by multiple signals including TCR. Egr1-deficient mice have poor accumulation of RTEs in the periphery, and this appears to originate from the decreased survival of mature thymocytes and RTEs [89]. AHR is a ligand-dependent member of the PAS-bHLH family of nuclear receptors. The overactivation of AHR leads to the preferential emigration of DN thymocytes and their accumulation in the spleen

[90]. Laminin-5 is expressed in the thymic medulla. Interactions of thymocytes with laminin-5 induced the release of a soluble fragment of surface molecule CD44, which leads to an increased migration of medullary thymocytes [91]. On the other hand, a defective expression of VLA-5 on NOD thymocytes was found to correlate with a decreased thymic output and a giant PVS filled with mature thymocytes [92].

Post-thymic maturation and peripheral tolerance of RTEs

The dynamics and homeostatic properties of RTEs

The idea that T cell development occurred only in the thymus was widely accepted until 1970s, when it was first proposed that T cells left the thymus in an immature state and completed their development in the periphery [93]. Subsequent studies further revealed that RTEs and peripheral mature naïve T cells are different both phenotypically and functionally [8,94]. The uniqueness of RTEs as a subpopulation different from SP thymocytes as well as mature naïve T cells has been gradually realized [8,94-99].

The proportion of RTEs to peripheral T cells changes during lifetime. In mice, RTEs occupy the entire peripheral T cell pool at 1-3 weeks of age. The ratio drops to 20% in young adult period, and further declines to 3% in 6-months old mice. But RTEs can be clearly detected in mice reaching 2-year of age [29]. RTEs are widely distributed in the periphery, as studies have found RTEs in the lymph nodes, Peyer's patches, spleen, blood and small intestine of mice [94,95,100]. Since RTEs and peripheral naïve T cells occupy some overlapping areas, researchers are eager to know which ones have the survival advantage over the other. Berzins et al. and Dong et al. reported that RTEs were preferentially incorporated into the peripheral T cell pool at the expense of their mature naïve T cell counterparts. In contrast, Houston et al. found that RTEs preferentially accumulated only in a lymphopenic environment due to their higher expression of CD24. In lymphoreplete mice, RTEs were disadvantaged competitors as both RTEs and naïve T cells had to compete for a limited survival niche [28,100-102]. Such discrepancy may come from different methods used to perform the experiments. Berzins et al. studied RTEs from hyperthymic (thymus-grafted) mice, whereas Dong et al. and Houston et al. performed adoptive transfer experiments with different subpopulations. Dong et al. compared the survival of CD4⁺ RTEs precursors (Qa2⁺CD69⁻ SP4 thymocytes) and CD4⁺ naïve T cells, and found that CD4⁺ pre-RTEs had the survival advantage over CD4⁺ naïve T cells in the periphery, while Houston et al. used CD4⁺ RTEs from the lymph nodes in the comparison and arrived at a different conclusion [28,100,101]. Thus, it is reasonable to think that RTEs purified from the lymph nodes have received maturation signals from the periphery and changed their homeostatic properties from their precursors in the thymus.

Methods in RTE studies

To facilitate the study of RTEs, several methods have been developed to distinguish them from other peripheral T cells. One method, direct intrathymic injection of FITC, can efficiently label murine thymocytes, enabling their subsequent identification in the peripheral lymphoid tissues. The advantage of this method is a direct phenotypic and functional study of the youngest RTEs by the analysis of peripheral FITC⁺ T cells. However, the surgical stress to the animals, limited time frame for detection (usually within 24 hours) as well as non-specific labeling of mature T cells recirculating to the thymus limit the wide application of this method [8,103,104]. The second method is giving the animals BrdU. Since BrdU is taken up by

dividing thymocytes, RTEs have been identified as BrdU^{lo}. However, this population is contaminated with post-division BrdU^{lo} mature T cells, blurring the distinction between RTEs and older peripheral T cells. Furthermore, detection of BrdU incorporation precludes functional studies [8,105]. The third method relies on T cell receptor rearrangement excision circles (TRECs). TRECs are stable and nonreplicative extrachromosomal circles of excised DNA during TCR gene recombination and are enriched in RTEs. However, not all TREC⁺ cells are RTEs since they can still be detected in the periphery after thymectomy; not all RTEs are TREC⁺ because only one daughter cell can get the excised DNA in division. Moreover, the TREC analysis is usually performed by real-time PCR, precluding the further phenotypic and functional characterization of this specific cell population [106-109]. Other methods such as thymic lobe grafts and fetal thymus organ culture were also used but they all have limitations such as creating an artificial full peripheral T cell compartment or introducing an in vitro system that may not accurately reflect the in vivo environment [5,8,96,100]. Some cell surface markers were also tested to define RTEs. For instance, mouse RTEs were defined as Qa2^{lo}CD24^{hi} and human RTEs were defined as CD31⁺PTK7⁺. Although this method allows for readily phenotypic and functional analysis of cells, whether these markers define the majority of peripheral RTEs was questioned [94,110,111].

Recently, the development of RAG1-GFP knockin and RAG2p-GFP transgenic mice made a big progress in RTE research. In these mice, the expression of GFP is driven by RAG1 or RAG2 promoter. After RAG gene expression is extinguished, the GFP signal remains detectable for a few more weeks, enabling the tracking of RTEs by GFP⁺ peripheral T cells [8,94]. According to the half-life of GFP protein, GFP^{hi} peripheral T cells have left the thymus within a week, GFP^{lo} ones are 1-2 weeks older and GFP⁻ cells represent the mature peripheral T cells [8,94]. This method allows the studies of live RTEs from unmanipulated mice. The residence time of RTEs in the periphery can be also indicated by the intensity of GFP signal. However, the intensity of GFP signal can be diluted by cell division. More importantly, even GFP^{hi} RTEs may have stayed in the periphery for a few days and may have received some regulation and made necessary alterations in the secondary lymphoid organs. It is thus hard to predict the immediate changes after thymic egress [8,94]. Despite these disadvantages, the RAG-GFP mice have been widely used in RTE research.

Post-thymic maturation of RTEs

RTEs are immature compared to peripheral naïve T cells, rendering post-thymic maturation necessary both phenotypically and functionally (Figure 1). Using RAG2p-GFP transgenic mice, the phenotypic analysis demonstrated that during their transition from GFP^{hi} to GFP^{lo} and finally GFP⁻, RTEs gradually down-regulated CD24 and CD3/TCR, and up-regulated Qa2, CD28, CD45RB, and IL-7R α [94,96,97]. Such pattern of phenotypic changes was supported by FITC and BrdU labeling of RTEs [20,97,105]. The changes of other surface markers during the maturation of RTEs include the upregulation of Ly6C, and higher levels of α 4 β 7, α E integrin and CCR9 expression in CD8⁺ RTEs than CD8⁺ naïve T cells [95,98,112,113].

In accordance with phenotypic differences, RTEs and naïve T cells are functionally distinct. Under non-polarizing conditions, activated CD4⁺ RTEs showed diminished proliferation when compared with CD4⁺ naïve T cells. This defect was only partly corrected by the addition of exogenous IL-2. Activated RTEs also secreted less IL-2, IL-4 and IFN- γ , expressed lower level of CD25

(IL-2 receptor α -chain) but similar level of CD69 [94,114,115]. The defect in IL-2 production was more obvious in aged mice [116]. Under Th1, Th17 and iTreg inducing conditions, RTEs expressed less characteristic cytokines or major transcription factors. On the contrary, more IL-4, IL-5 and IL-13 were produced both in vivo and in vitro in Th2-polarized RTEs when compared with Th2-polarized naïve T cells [99,115,117]. The ex vivo analysis of transcription factor and cytokine receptor expression suggested that instead of biased towards the Th2 cell lineage, CD4⁺ RTEs are biased away from the Th1 cell lineage [115]. Compared with CD8⁺ naïve T cells, CD8⁺ RTEs contain a lower frequency of cytolytic precursors, suggesting the existence of functional defects. Indeed, CD8⁺ RTEs secreted less tumor necrosis factor (TNF) with anti-CD3 and anti-CD28 stimulation in vitro [118]. After bacterial or viral infections, activated CD8⁺ RTEs produced less cytokines and generated fewer IL-7R α^{hi} KLRG1 $^{\text{lo}}$ memory precursor effector cells [119,120].

The phenotypic and functional maturation process is a result of maturation at the single cell level, not selective survival and proliferation of a small population of relatively mature RTEs [60]. This leads investigators to explore the mechanisms of post-thymic maturation. First, thymic egress is required for the acquisition of a complete phenotypic maturation because mice treated with blocking antibodies such as AAL-R (a synthetic mimetic of S1P) to inhibit thymic egress showed an immature phenotype of RTE candidates [60]. Second, the phenotypic and functional maturation also requires the access to secondary lymphoid organs, as a combination of splenectomy and administration of anti-CD62L plus anti-VLA-4 to block lymph node entry also impaired RTE maturation [60]. Further studies revealed that a full dendritic cell compartment in the secondary lymphoid organs was indispensable for phenotypic maturation of RTEs while self-peptide-MHC complexes and IL-7 were dispensable [121]. The transcriptional repressor NKAP may also influence RTE maturation, as NKAP deficiency keeps RTEs from full maturation [122]. However, up to now, the mechanism of RTE maturation at the molecular level remains largely unknown.

Peripheral tolerance of RTEs

Although central tolerance can eliminate most of the autoreactive T cells in the thymus, some of them inevitably escape and export to the periphery, rendering the necessity of peripheral tolerance to these RTEs [123,124]. Complementarity determining region 3 (CDR3) length spectratyping revealed that TCRs expressed by RTEs were skewed toward longer CDR3 regions compared with naïve T cells, suggesting the existence of more autoreactive cells in the RTE population [125-127]. The mechanisms of peripheral tolerance of RTEs are obscure with scattered evidence revealing some possible explanations. CD8⁺ RTEs could enter neonatal nonlymphoid tissues such as skin and become tolerized to antigens expressed there [128]. Higher level of $\alpha 4\beta 7$, αE integrin, and CCR9 on CD8⁺ RTEs facilitated their homing to the gut-associated lymphoid tissues. This pattern of migration may allow intestine-homing RTEs to gain tolerance to self-antigens and harmless food antigens there [95,98,111,113]. Moreover, RTEs appeared to be tolerized in vivo by alloantigen, as RTEs failed to cause graft-versus-host disease (GVHD) when transferred to allogeneic mice [96]. The diminished proliferation and defective cytokine secretion may contribute to their peripheral tolerance [8,94]. Except for selective migration and immunoincompetence of RTEs mentioned above, self-antigens presented by DCs and lymphoid stroma in the periphery may also promote peripheral tolerance [129,130]. Another important

mechanism is the expression of some inhibitory receptors by RTEs, such as CTLA-4 and PD-1. Severe autoimmune disease was found in Rag^{-/-} mice when transferred with RTEs deficient in PD-1 [96,131].

Summary and Outlook

Previous studies have suggested that instead of simply waiting for export to the periphery, SP thymocytes undergo multiple stages of maturation in and outside of the thymus before they become mature naïve T cells. This process is under precise regulation of thymic and peripheral microenvironment. As RTEs were found enriched in several disease tissues, such as ulcerative colitis and chronic myeloid leukaemia [132,133], it is reasonable to hypothesize that these diseases may affect the maturation of SP/RTEs and defective RTEs may play a pathological role in disease progression. Compared to our knowledge in the early stage of thymocyte development, the process of SP/RTE/Naïve T cell transition is far from being well understood. Its underlying mechanisms and its relationship to diseases should be further explored.

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The authors have declared that no competing interests exist.



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