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Thymic emigration: when and how T cells leave home[1]

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Abstract

The thymus supports the differentiation of multiple distinct T cell subsets that play unique roles in the immune system. CD4 and CD8 α/β T cells, γ/δ T cells, NKT cells, T_{reg}, and IEL all develop in and must leave the thymus to provide their functions elsewhere in the body. This article will review recent research indicating differences in the time and migration patterns of T cell subsets found in the thymus. Additionally we review current understanding of the molecules involved in thymocyte emigration, including the sphingolipid receptor, $S1P_1$, and its regulation by the transcription factor KLF2.

Introduction

The thymus is a unique site for development of T lymphocytes. It provides an inductive environment for T lineage commitment and V(D)J recombination at the γδ and αβ TCR loci. Most important, it provides a specialized environment for the selection of rearranged clones that will function appropriately in the adaptive immune system. This includes positive selection of T cells with appropriate MHC restriction, negative selection against self-reactivity, and development of unique regulatory properties in subsets such as NKT, T_{reg}, and IEL. Thus, to date, the thymus is known to export at least six different populations of T cells: γ/δ T cells, naïve CD4 and CD8 α/β T cells, NKT cells, T_{reg}, and IEL or IEL progenitors. This review will concentrate on when, where, and how T cells emigrate from the thymus.

When do thymocytes emigrate?

For conventional T cells, positive selection on either MHC Class I or II induces the differentiation of a DP progenitor into a CD8 or CD4 SP, respectively (see Figure 1). Coincident with this, they upregulate CCR7 and migrate to the medulla (1,2). In thinking about the timing of emigration, it is important to consider when a given progenitor achieves functional maturity. Directly after positive selection SP thymocytes have a “semi-mature” cell surface phenotype (Qa-2^{low}, CD62L^{low}, HSA^{hi}, $CD69^{hi}$) that is associated with susceptibility to apoptosis (3) (see Figure 1). As the cell differentiates, its surface phenotype changes to Qa-2^{hi}, CD62L^{hi}, HSA^{low}, $CD69^{low}$. Although this ordered differentiation was inferred through multiple approaches in the past, a study published this year used an elegant direct approach to document the changes (4). They sorted four different SP subsets and CFSE labeled them prior to intrathymic injection into recipients. They were able to confirm the order and timing of SP differentiation, and to show that most thymocytes emigrate in the

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absence of cell division. This is interesting, as previous studies had suggested that some or all SP thymocytes divide at least once (5). The phenotypic changes that define a “mature” SP thymocyte are associated with an important difference in function—the progenitor is no longer susceptible to apoptosis, and instead proliferates if triggered through the antigen receptor. We recently showed that only this proliferation competent “mature” SP subset emigrates from the thymus (6).

Although the T cells that emigrate from the thymus are proliferation competent, such T cells may not be fully mature at this point. Fink and colleagues pioneered the use of Rag2p-GFP mice to define and study recent thymic emigrants (7). In these mice, GFP is expressed at a high level in DP thymocytes, where Rag2 is strongly expressed. Although Rag2 itself is rapidly repressed after positive selection, GFP lingers as it is a relatively stable protein. Thus cells that recently emigrated from the thymus can be identified by a “shoulder” of GFP expression, which decays with a predictable kinetic after thymectomy (7). Using this approach, they showed that after leaving the thymus, T cells continue to upregulate Qa-2 and down-regulate HSA. Furthermore recent thymic emigrants displayed reduced capacity for proliferation, CD25 upregulation, and IL-2 secretion, when compared to the bulk population of naïve T cells. It is not yet known what cellular and molecular factors control the final thymic and extrathymic maturation step, and if they are distinct, or extensions of the same process.

To understand how long thymic emigration takes in real time, we recently determined the half-life of GFP protein in such RAG2p-GFP transgenic mice. By comparing the GFP levels on DP thymocytes and emigrants that just left the thymus, we showed that conventional αβ T cells emigrate after a maximum of 4–5 days (6). 4–5 days is an upper estimate since we did not take proliferation of SPs into account and proliferation would also decrease GFP levels. This is in contrast to what was previously suggested about the timing of emigration based on a continuous labeling approach (8,9), however these studies were complicated by heterogeneity of the SP pool (see below). When excluding non-conventional and memory T cells from the analysis, we found that the continuous labeling approach also suggested emigration occurs after only 4 days.

The time spent in the thymic medulla is crucial for SP thymocytes to become self-tolerant, because medullary epithelial cells (mECs) express tissue specific antigens (TSA) (10). This ability is endowed in part by the transcription factor Aire, which is restricted to mECs (11). Aire-deficient mice and humans develop autoimmunity (12), as do mice with defective development of mEC (13). Thus without migrating to the medulla, thymocytes specific for TSA mature into peripheral T cells and mediate disease (14). Since not every mEC expresses every TSA (15), it follows that careful timing of emigration is necessary to allow semi-mature SPs to interact with as many medullary APCs as possible to increase the efficiency of deleting self-reactive thymocytes. Although SP thymocytes spend up to 4 days in the medulla, they are in an apoptosis susceptible state for only about half of this time. Thus, two days is presumably sufficient to allow for interactions with medullary APC for tolerance induction.

Non-conventional T cells comprise part of the SP thymocytes pool

As discussed above, our laboratory found the time between positive selection and emigration to be much shorter than previously believed for αβ thymocytes. This is because previous analysis included NKT, T_{reg}, γδ, and recirculating memory αβ T cells, which all are included in the “thymic SP” population. Such cells typically comprise 10–15% of the SP pool in young (6–11 week old) mice, and this increases with age (unpublished data). For conventional αβ T cells, only activated/memory T cells recirculate to the thymus in adults, and can be identified by phenotype (16). However, the phenotype of the recirculating non-

conventional T cells is not well established, complicating analysis. Using the Rag2p-GFP mice, in which GFP decay correlates with age, non-conventional subsets can be much lower for GFP indicating either thymic retention, increased cell division, or recirculation back to the thymus (6). We did not find a high rate of proliferation in T_{regs}, thus the fact that 50% of thymic T_{reg} are GFP low/negative may reflect recirculation back to the thymus. Indeed, Ceredig and colleagues used parabiosis to show that circulating T_{reg} could return to the thymus, although this was most pronounced in lymphopenic situations (17). By depleting peripheral T_{regs}, Lew and colleagues estimated that at least 20% of thymic T_{regs} have recirculated from the periphery (18). A different story is found for NKT cells. Many NKT cells express a semi-invariant TCR and can be identified using $CD1^d$/αGal-cer tetramers. In the thymus, NK1.1− (immature) NKT give rise to NK1.1+ (mature) NKT (19). Interestingly, the NKT that emigrate from the thymus are NK1.1− (19,20). Furthermore, Godfrey and colleagues found using thymus transplants that mature, NK1.1+ do not migrate and are retained in the thymus for over one year (21). Thus the low level of GFP in virtually all of the thymic NK1.1+ NKT likely reflects their lengthy retention in the organ.

What are the sites of emigration?

The transition from semi-mature to mature SP occurs in the medulla but via what structures do mature thymocytes actually leave? The blood vascular structure of the thymus is such that major arteries and veins enter and leave via the septae, and articulate out at the cortico-medullary junction with capillaries looping out into the cortex (22). Indeed progenitors enter through venules in the cortico-medullary junction (23). The thymus also has efferent lymphatics, although their structure and development is less well understood. There is evidence to support both lymphatic and blood emigration routes (24,25). In images of the thymus one can find examples of mature thymocytes “lined up” inside perivascular spaces along the blood vessels in the cortico-medullary junction and nearby medulla (26). Diapedisis through the endothelium into the blood through post-capillary venules has been observed (27). The connections between the perivascular space and lymphocyte rich lymphatic vessels have also been observed and postulated as an exit route (28). Indeed emigrating T cells have been found in the lymph draining from cervical thymuses of guinea pigs and lambs (25,29). Recently, Pappu *et al*. evaluated this issue using mice with genetic deficiency in the generation of S1P, a spingolipid ligand that is crucial for emigration (discussed in detail below). The redundant sphingosine kinases *Sphk1* and *Sphk2* are necessary for S1P synthesis and S1P is detected at high levels in both blood and lymph. Using bone marrow chimeras, they were able to determine that lymph S1P is radioresistent in origin (possibly endothelial) and blood S1P is hematopoietic (dependent on red blood cells) (30). When lymph S1P was depleted, there was a 50% increase in accumulation of mature CD4s over control. Although this was significant, it was much less than the 4-fold increase seen in intact S1P KO animals. The opposite chimeras showed decreased S1P concentration in the blood but the gradient remained high enough to support egress. Thus, the partial emigration defect supports a model where thymocytes exit into both the blood and lymph. It is possible that different subsets of emigrating T cells have distinct blood/lymphatic preference for emigration, although this has not been studied.

Thymocytes normally mature and exit from the medulla or cortico-medullary junction but medullary migration is not necessarily a prerequisite for emigration. As mentioned above, CCR7 upregulation after positive selection mediates thymocyte migration to the medulla. CCR7- or CCR7L-deficient mice generate SP thymocytes that do not traffic to the thymic medulla (2). Nonetheless, CCR7-deficient thymocytes are able to emigrate, apparently directly from the cortex. Treatment with FTY720, an inhibitor of thymocyte emigration discussed later, led to accumulation of CCR7-WT SPs in medullary perivascular spaces and the accumulation of CCR7-deficient SPs in cortical perivascular spaces (14). Thus, the

perivascular space and vessels of the medulla and cortico-medullary junction may not be specialized for thymocyte egress but may simply be the vessels used under steady state circumstances because of their proximity to mature thymocytes.

Cell surface molecules involved

Until fairly recently very little was known about the receptors, ligands, and molecular signals necessary for thymocyte emigration. Earlier work showed that expression of the catalytic subunit of pertussis toxin in thymocytes inhibited their emigration pointing toward the involvement of a $G\alpha_i$ protein coupled receptor (GPCR) (31). Studies of egress from fetal thymic organ cultures suggested that CCR7 plays a role in the neonatal period (32), but in the adult, thymocytes emigrate independently of CCR7, despite having an impaired cortex to medulla migration (1,2,14). Chemorepulsion or fugetaxis has also been proposed to have a role in thymic emigration. In *in vitro* assays, CXCR4-expressing thymocytes move away from the thymus in a SDF-1 dependent manner (33). The disruption of SDF-1/CXCR4 interactions by genetic deficiency or pharmacological antagonism, with AMD3100, led to decreased migration in fetal thymic organ culture. *In vivo* treatment with AMD3100 in newborn mice lead to accumulation in the thymus of CD4 SP and less CD4s in spleen and but no difference in lymph node number (34). Emigration of CD8s was not affected so CD4 and CD8 may differ in their thymic emigration requirements, at least in the neonatal period.

A breakthrough in our knowledge of adult thymic emigration came with development of the immunosuppressive drug FTY720 and discovery of its mechanism of action. A screen of fungal metabolites found that myriocin had lymphocyte-specific immunosuppressive effects. Myriocin effects were 10x more potent than cyclosporine in a mixed lymphocyte reaction and apparently by a different mechanism because IL-2 secretion was not affected (35). The *in vivo* toxicity of myriocin led to the search for less toxic derivatives. FTY720 was the most promising candidate retaining the immunosuppressive function with less toxicity (36). FTY720 treatment in animal models prevented graft rejection and depleted lymphocytes from blood (37). The mechanism of the lymphopenia is sequestration of T cells in lymph nodes by inhibiting egress from those tissues. Further work showed that FTY720 also profoundly blocks egress of thymocytes from the thymus (38–40). Another imidazole based compound that was shown to block thymic emigration (41) was later discovered to be an inhibitor of S1P lyase (42). Thus it inhibits emigration by disrupting the S1P gradient between lymphoid tissue, including the thymus, and the blood and lymph (42).

FTY720 is structurally similar to the lysophospholipid sphingosine. FTY720 is phosphorylated *in vivo* and phosphorylated FTY720 and sphingosine-1-phosphate (S1P) are the molecules that produce the *in vivo* effects on T cell trafficking (43). There are five G-protein coupled receptors specific for S1P and phosphorylated FTY720 is an agonist for four: $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ (43). There is significant expression of $S1P_1$ and $S1P_4$ on CD4 and CD8 T cells (44). Knockout of $S1P_1$ results in an embryological death from vascular defects (45). When chimeras were made from $S1P_1$ −/− fetal livers, T cells were nearly absent from all peripheral lymphoid organs despite relatively normal thymic development (46,47). Mature $S1P_1$ −/− thymocytes accumulate in the thymus as a result of decreased emigration. $S1P_1$ −/− SP thymocytes have a mature, CD62L high, HSA low, β7 integrin high phenotype. However, the expression of CD69, which normally is downregulated as SPs mature, remained high (46).

This puzzling failure of CD69 to become downregulated can be explained by the recent work suggesting that CD69 and $S1P_1$ mutually antagonize each other in a post-translational fashion. The over-expression of one on the cell surface leads to the down regulation of the other (46). In the case of $S1P_1$ −/− SPs with no $S1P_1$ to antagonize its cell surface expression CD69 remains on the cell surface. This $S1P_1$/CD69 interactions would neatly

explain the observation that CD69 transgenic thymocytes have a thymic emigration defect (48). In this case the overexpression of CD69 would cause the downregulation of $S1P_1$ from the surface resulting in failure to egress. The experiments to date do not rule out that CD69 has an $S1P_1$ independent retention effect as well though.

In addition to inhibiting lymph node egress and thymic emigration, FTY720 also inhibits T cell emigration out of inflamed tissues (49). $S1P_1$ is also found on endothelial cells and FTY720 affects junctions of the endothelium (50). It is possible the effects of $S1P_1$ antagonism on the endothelium could mediate the changes in T cell egress from thymus or lymph nodes (50). Nonetheless, T-cell specific $S1P_1$ deficiency alone results in a thymic emigration defect, so the T cells themselves are an important, albeit perhaps not exclusive, target of FTY720 and $S1P_1$ deficiency (46,47,51).

Transcriptional regulation of the emigration process

Knowledge of the receptors necessary for emigration from the thymus brings up the question of what controls the expression of these receptors. Earlier work showed that transgenic expression of the transcription factor *FoxJ1* inhibits thymic emigration (52). The underlying mechanism is unknown but seems to be $S1P_1$ independent. Our laboratory found that the transcription factor Kruppel Like Factor 2 (KLF2) regulates T cell expression of $S1P_1$ and thymocyte emigration (53). Like $S1P_1$ −/− mice, KLF2 −/− mice die before birth of hemorrhaging caused by circulatory defects (54,55). Thus to study KLF2 deficiency in the hematopoietic lineages, RAG −/− blastocyst chimeras, fetal liver chimeras, or conditional deficiency have been employed. When the bone marrow lacks KLF2, most hematopoeitic lineages appear normal, except for a striking loss of T cells from secondary lymphoid organs (53,56). The thymus is approximately normocellular without a block in development. However, there is a preferential accumulation of mature thymocytes, and a dramatic reduction in the number of recent thymic emigrants detected using intrathymic injection of a covalent label (53). Likewise, using the above mentioned Rag2p-GFP mice, we observed that KLF2 deficient SP are retained in the thymus substantially longer than normal (57).

It is likely that KLF2 directly regulates transcription of $S1P_1$. Both $S1P_1$ mRNA and surface expression are reduced in KLF2 KO (53,58,59). There is a KLF2 consensus sequence in the putative $S1P_1$ promoter region, and chromatin immunoprecipitation showed a direct interaction of KLF2 with the $S1P_1$ promoter (53). As in the $S1P_1$ KO, CD69 surface expression remains high on KLF2 deficient thymocytes, without a change in mRNA expression, suggesting that CD69 alterations are secondary to the $S1P_1$ loss (unpublished data). However, KLF2 KO thymocytes differ from $S1P_1$ KO thymocytes in the dysregulation of at least one other important cell surface molecule--CD62L. CD62L, or L-selectin, is not required for thymocyte emigration, but it is required for entry from circulation into lymph nodes. Evidence suggests that KLF2 directly regulates CD62L, as it does $S1P_1$ (58). Thus it would appear that KLF2 coordinates the expression of genes that are vital to the ability of naïve T cells to circulate through secondary lymphoid organs (60).

Very recently, Sebzda *et al.* published an alternative explanation for the paucity of T cells in the secondary lymphoid organs of KLF2 deficient mice (59). This group used Vav-cre and a floxed KLF2 to create T cell specific deficiency of KLF2. They proposed that KLF2 −/− thymocytes do not have an intrinsic thymic emigration defect but rather leave the thymus normally and are rapidly sequestered in tissues due to dysregulation of chemokine receptors (59). A key piece of data in their study was the finding that the S1P agonist FTY720 caused an increase in the number of T cells that could be recovered from secondary lymphoid organs of KLF2−/− mice. They interpreted this to mean that KLF2 deficiency did not lead to a complete loss of $S1P_1$. However FTY720 may be acting on T cell $S1P_4$ or on endothelial $S1P_1$, both of which are expressed in their system. Their interpretation also does

not explain the increased retention of mature thymocytes within the thymus, which is a striking aspect of the KLF2−/− mouse, phenocopied precisely in the $S1P_1$−/− mouse. Furthermore, we do not observe a preferential accumulation of KLF2−/− αβ T cells in non-lymphoid tissues (57). For these reasons, we favor the idea that the primary defect in KLF2−/− mice is impaired thymic emigration due to loss of $S1P_1$. Nonetheless, the idea that KLF2 also represses chemokine receptor gene expression is fascinating, and further suggests KLF2 may be a master regulator of T cell migration.

How does a T cell know when to go?

An interesting question that remains is what ultimately regulates KLF2 and emigration from the thymus. The prevention of autoimmunity by negative selection to tissue specific antigens after positive selection is predicated on retention of SP thymocytes in the medulla. KLF2 and $S1P_1$ are not highly expressed until at least 4 days after positive selection (6). So, what signals induce the upregulation of these molecules if not positive selection, and if it is positive selection, why is gene expression delayed for days? Sohn *et al.* found that ERK5 signaling leads to KLF2 induction in embryos (61). They also found that IL-7 stimulation caused the phosphorylation of ERK5, suggesting a model in which IL-7 signaling activates ERK5 and induces KLF2 expression. IL-7 signals are actively repressed in DP thymocytes and after positive selection thymocytes regain responsive (62), which could explain the timing of KLF2 expression. However we found T cells with conditional deficiency in ERK5 (63) had no defect in KLF2 expression or thymic emigration. Nor did IL-7R blockade alter KLF2 expression and thymic emigration (data not shown). Thus, the signaling pathways that direct developing thymocytes to emigrate remain enigmatic.

Interestingly, naïve T cells rapidly terminate KLF2 gene expression when activated by antigen (56,64). Sinclair *et al.* recently demonstrated the phosphatidylinositol-3-OH kinase (PI3K) signaling in T cells triggers KLF2 loss (65). KLF2 is re-expressed when cultured in IL-15 while IL-2, which strongly activates PI3K signaling, continues the repression. In T cells deficient for PTEN, a PI3K negative regulator, there is an accumulation of mature, HSA low thymocytes, consistent with a defect in emigration (65). Transgenic mice expressing the catalytic subunit of class I_A PI3K results in active PI3K signaling and a defect in emigration of thymocytes to the periphery, which may be due to decreased KLF2 expression (66). Likewise, activation of mature T cells directly in the thymus impairs their emigration (67) probably via a similar mechanism.

Concluding Remarks

While selection of T cells clones at the DP stage have been intensively studied, more recent research highlights the importance of post-positive selection maturation. In particular, negative selection to TSA in the medulla is required to prevent autoimmunity. T cells also undergo functional maturation late in their development. To allow both of these processes to occur, thymic emigration must be tightly regulated. Although emigration is still incompletely understood, recent work has defined key molecules, such as the transcription factor KLF2 and the cell surface molecule $S1P_1$, which are vital to this process and will be a focus for future research.

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Abbreviations used in the paper

TSA	tissue specific antigens
mEC	medullary epithelial cell
GPCR	$G\alpha_i$ protein coupled receptor
KLF2	Kruppel Like Factor 2
PI-3K	phosphatidylinositol-3-OH kinase

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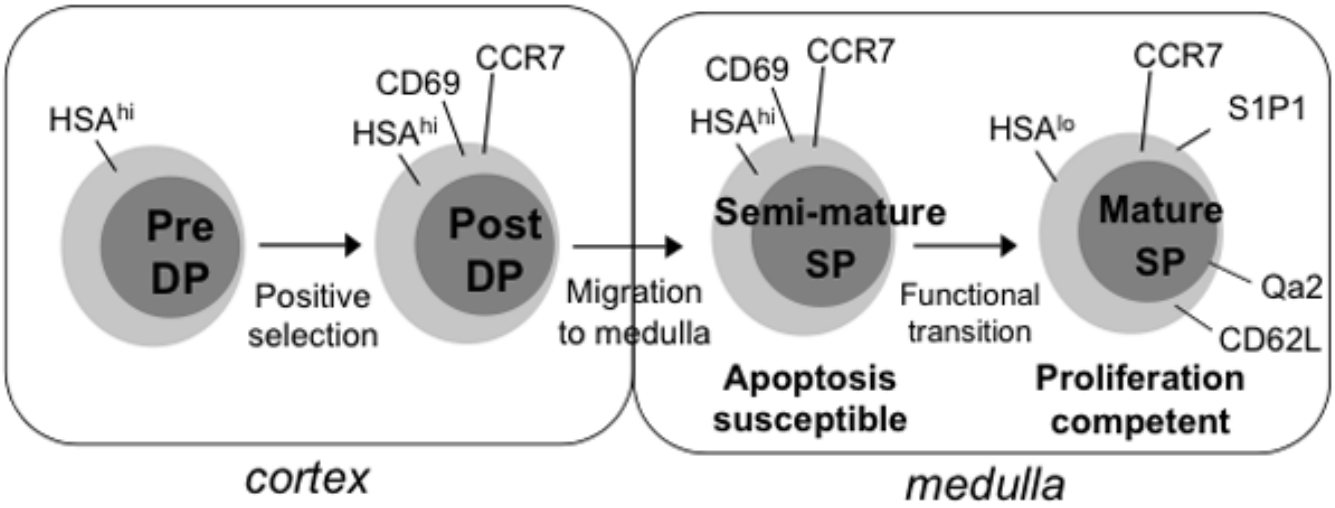

Figure 1. Developmental events that occur after positive selection in the thymus

Prior to positive selection HSA^{hi} DP thymocytes reside in the cortex. Upon interaction with selecting MHC, they upregulate CD69 and CCR7 and migrate to the medulla, commensurate with becoming an SP. Semi-mature SP thymocytes remain susceptible to apoptosis, which is presumably critical for tolerance to tissue specific antigens displayed by APC in the medulla. Ultimately the progenitor undergoes final functional maturation, after which it is competent to proliferate when triggered through the TCR. At this stage it upregulates Qa2, CD62L, and S1P1, and emigrates.

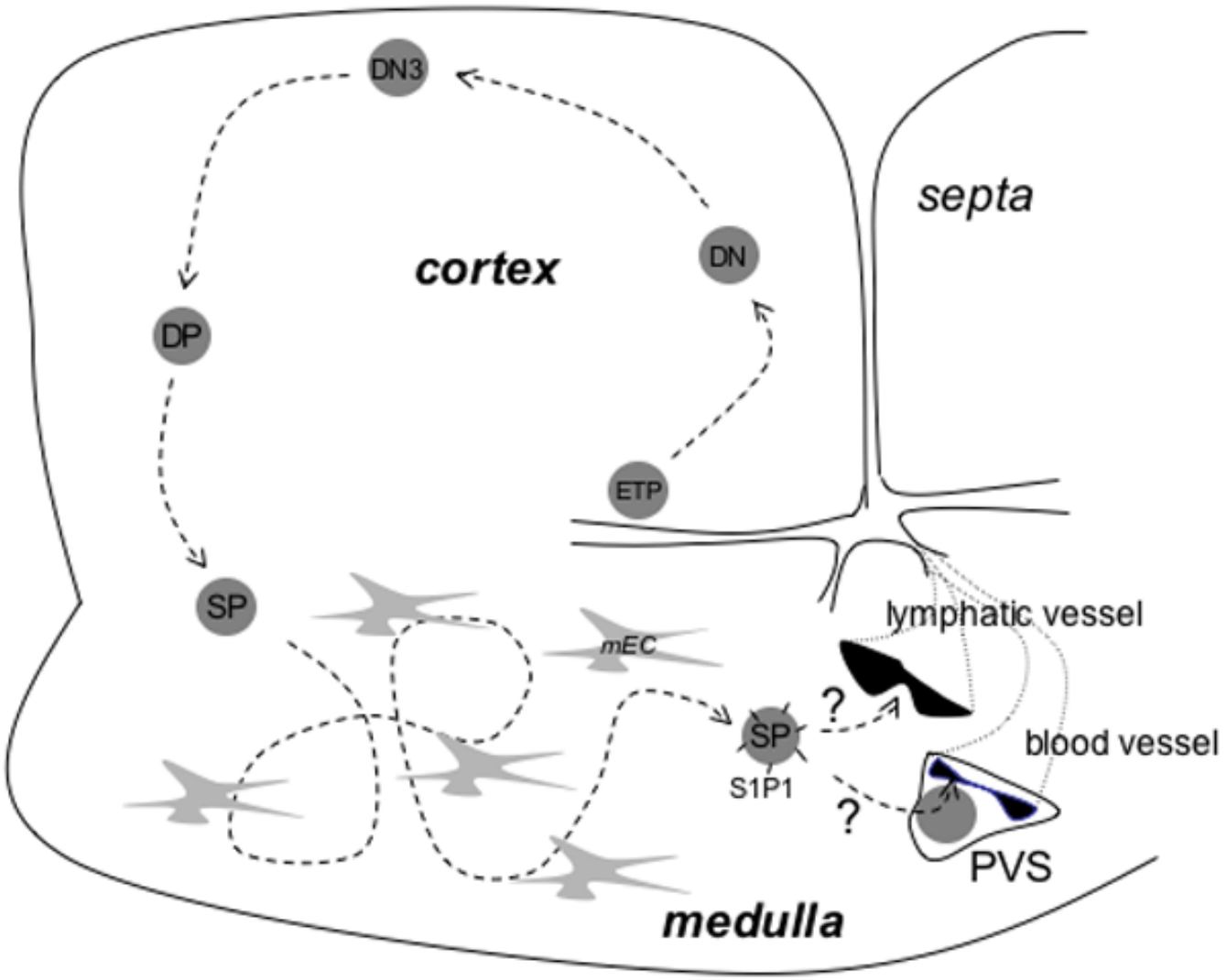

Figure 2. Overview of progenitor movement through the thymus

Multipotent progenitors are recruited into the thymus from the blood at the cortico-medullar junction. They transmigrate the cortex in the early DN stages, residing near the outer capsule at the DN3 stage. As cells progress to the DP stage they begin a reverse migration back through the cortex where they interact with cortical epithelial cells for positive selection. As cells differentiate to the SP stage after positive selection, they migrate to the medulla. SP thymocytes spend up to four days perusing the medulla. For the first half of this time period, they are in an apoptosis susceptible state. After a final functional maturation, they upregulate $S1P_1$, a cell surface molecule that facilitates movement into the circulation. Thymocytes likely enter the circulation via lymphatics or blood vessels in the medulla.