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The thymus supports the differentiation of multiple distinct T cell subsets that play unique roles in the immune system. CD4 and CD8 αβ T cells, γδ T cells, NKT cells, regulatory T cells, and intraepithelial lymphocytes all develop in the thymus and must leave it 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, and its regulation by the Krüppel-like transcription factor KLF2. "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 that decays with a predictable kinetic after a thymectomy (7). Using this approach, they showed that after leaving the thymus, T cells continue to up-regulate Qa-2 and down-regulate heat-stable Ag (HSA). Furthermore, recent thymic emigrants displayed reduced capacity for proliferation, CD25 up-regulation, and IL-2 secretion when compared with the bulk population of naive T cells. It is not yet known what cellular and molecular factors control the final thymic and extrathymic maturation step and whether 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 the 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). Four to 5 days is an upper estimate because we did not take the proliferation of SP thymocytes 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 the heterogeneity of the SP pool (see below). When excluding nonconventional and memory T cells from the analysis, we found that the continuous labeling approach also suggested that 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 Ags (TSAs) (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 mECs (13). Thus, without migrating to the medulla thymocytes specific for TSA mature into peripheral T cells and mediate disease (14). Because not every mEC expresses every TSA (15), it follows that careful timing of emigration is necessary to allow semimature 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, 2 days is presumably sufficient to allow for interactions with medullary APCs for tolerance induction.

**Nonconventional T cells comprise part of the SP thymocyte 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, Treg, γδ, and recirculating memory αβ T cells, all of which are included in the “thymic SP” population. Such cells typically comprise 10–15% of the SP pool in young (6–11 wk old) mice, and this increases with age (our 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 nonconventional T cells is not well established, complicating analysis. Using the Rag2p-GFP mice in which GFP decay correlates with age, nonconventional subsets can be much lower for GFP, indicating thymic retention, increased cell division, or recirculation back to the thymus (6). We did not find a high rate of proliferation in Treg cells; thus, the fact that 50% of thymic Treg are GFPlow or GFP− may reflect recirculation back to the thymus. Indeed, Cerdec and colleagues used parabiosis to show that circulating Tregs could return to the thymus, although this was most pronounced in lymphopenic situations (17). By depleting peripheral Treg, Lew and colleagues estimated that at least 20% of thymic Treg 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 CD1dα-galactosylceramide tetramers. In the thymus, NK1.1− (immature) NKTs give rise to NK1.1+ (mature) NKT (19). Interestingly, the NKTs that emigrate from the thymus are NK1.1+ (19, 20). Furthermore, Godfrey and colleagues found using thymus transplants that mature, NK1.1+ cells do not migrate and are retained in the thymus for >1 year (21). Thus, the low level of GFP in virtually all of the thymic NK1.1+ NKT cells likely reflects their lengthy retention in the organ.

**What are the sites of emigration?**

The transition from semimature to mature SP thymocytes 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 corticomedullary junction, with capillaries looping out into the cortex (22). Indeed progenitors enter through venules in the corticomedullary junction (Ref. 23 and see Fig. 2). The thymus also has efferent lymphatics, although their structure and development are 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 corticomedullary junction and nearby medulla (26). Diapedesis through the endothelium into the blood through postcapillary venules has been observed (27). The connections between the perivascular space and the lymphocyte-rich lymphatic vessels have also been observed and postulated as exit routes (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 sphingosine-1-phosphate (SIP), a sphingolipid ligand that is crucial for emigration (Ref. 30; discussed in detail below). The redundant sphingosine kinases Sphk1 and Sphk2 are necessary for SIP synthesis, and SIP is detected at high levels in both blood and lymph. Using bone marrow chimeras, Pappu et al. were able to determine that lymph SIP is radioreistant in origin (possibly endothelial) and that blood SIP is hematopoietic (dependent on RBC) (30). When lymph SIP was depleted, there was a 50% increase in the accumulation of mature CD4 over control. Although this was significant, it was much less than the 4-fold increase seen in intact SIP knockout (KO) animals. The opposite chimeras showed decreased SIP 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 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 corticomedullary junction but medullary migration is not necessarily a prerequisite for emigration. As mentioned above, CCR7 up-regulation after positive selection mediates thymocyte migration to the medulla. CCR7- or CCR7 ligand-deficient mice generate SP thymocytes that do not traffic to the thymus (38 – 40). Another imidazole-based compound that was shown to block thymic emigration (41) was later discovered to be an inhibitor of SIP lyase (42). Thus, it inhibits emigration by disrupting the SIP 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 SIP are the molecules that produce the in vivo effects on T cell trafficking (43). There are five G protein-coupled receptors specific for SIP, and phosphorylated FTY720 is an agonist for four of them: SIP1, SIP3, SIP4, and SIP5 (43). There is significant expression of SIP1 and SIP4 on CD4 and CD8 T cells (44). Knockout of SIP1 results in an embryological lethality from vascular defects (45). When chimeras were made from SIP1–/– fetal livers, T cells were nearly absent from all peripheral lymphoid organs despite relatively normal thymic development (46, 47). Mature SIP1–/– thymocytes accumulate in the thymus as a result of decreased emigration. SIP1–/– SP thymocytes have a mature, CD62Lhigh/CD44low/β2-integrinhigh phenotype. However, the expression of CD69, which normally is down-regulated as SPs mature, remained high (46).

This puzzling failure of CD69 to become down-regulated can be explained by the recent work suggesting that CD69 and SIP1 mutually antagonize each other in a posttranslational fashion. The overexpression of one on the cell surface leads to the down-regulation of the other (46). In the case of SIP1–/– SP with no SIP1 to antagonize its cell surface expression, CD69 remains on the cell surface. This SIP1/CD69 interaction 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 down-regulation of SIP1 from the surface, resulting in failure to egress. The experiments to date do not rule out that CD69 has an SIP1-independent retention effect as well, however.

In addition to inhibiting lymph node egress and thymic emigration, FTY720 also inhibits T cell emigration out of inflamed tissues (49). SIP1 is also found on endothelial cells and pharmacological antagonism with AMD3100 led to decreased migration in fetal thymic organ culture. In vivo treatment with AMD3100 in newborn mice led to accumulation in the thymus of CD4 SP and fewer CD4 T cells in spleen but no difference in lymph node number (34). Emigration of CD8 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 10× more potent than cyclosporine in a MLR 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 SIP lyase (42). Thus, it inhibits emigration by disrupting the SIP gradient between lymphoid tissue, including the thymus, and the blood and lymph (42).

In contrast, thymocytes normally mature and exit from the medulla or corticomedullary junction but medullary migration is not necessarily a prerequisite for emigration. As mentioned above, CCR7 up-regulation after positive selection mediates thymocyte migration to the medulla. CCR7- or CCR7 ligand-deficient mice generate SP thymocytes that do not traffic to the thymus (38–40). Another imidazole-based compound that was shown to block thymic emigration (41) was later discovered to be an inhibitor of SIP lyase (42). Thus, it inhibits emigration by disrupting the SIP gradient between lymphoid tissue, including the thymus, and the blood and lymph (42).
FTY720 affects junctions of the endothelium (50). It is possible the effects of S1P, antagonism on the endothelium could mediate the changes in T cell egress from thymus or lymph nodes (50). Nonetheless, T cell-specific S1P, 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, 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, independent. Our laboratory found that the transcription factor Krüppel-like factor 2 (KLF2) regulates T cell expression of S1P, and thymocyte emigration (53). Like S1P,−/− mice, KLF2−/− mice die before birth from hemorrhaging caused by circulatory defects (54, 55). Thus, to study KLF2 deficiency in the hematopoietic lineages, RAG−/− blastocyst chimeras, FL chimeras, or conditional deficiency have been used. When the bone marrow lacks KLF2, most hematopoietic 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 thymocytes are retained in the thymus substantially longer than normal (O. Odumade, A., K. Takada, M. A. Weinreich, T. M. McCaughtry, J. Huddleson, J. Neumann, J. B. Lingrel, S. C. Jameson, and K. A. Hogquist, The role of KLF2 in maturation and thymic emigration of naive and nonconventional T cell lineages, submitted for publication.).

It is likely that KLF2 directly regulates transcription of S1P,. Both S1P, mRNA and surface expression are reduced in KLF2 KO thymocytes (53, 57, 58). There is a KLF2 consensus sequence in the putative S1P, promoter region, and chromatin immunoprecipitation showed a direct interaction of KLF2 with the S1P, promoter (53). As in the S1P,−/− thymocytes, CD69 surface expression remains high on KLF2-deficient thymocytes without a change in mRNA expression, suggesting that CD69 alterations are secondary to the S1P, loss (our unpublished data). However, KLF2 KO thymocytes differ from S1P,−/− 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, (57). Thus, it would appear that KLF2 coordinates the expression of genes that are vital to the ability of naive T cells to circulate through secondary lymphoid organs (59).

Very recently, Sebzda et al. published an alternative explanation for the paucity of T cells in the secondary lymphoid organs of KLF2-deficient mice (58). 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 (58). 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,. However FTY720 may be acting on T cell S1P, or on endothelial S1P, 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,−/− mouse. Furthermore, we do not observe a preferential accumulation of KLF2−/− αβ T cells in nonlymphoid tissues (Odumade, O. A., K. Takada, M. A. Weinreich, C. M. Carlson, T. M. McCaughtry, J. Huddleson, J. Neumann, J. B. Lingrel, S. C. Jameson, and K. A. Hogquist, The role of KLF2 in maturation and thymic emigration of naive and nonconventional T cell lineages, submitted for publication.). For these reasons, we favor the idea that the primary defect in KLF2−/− mice is impaired thymic emigration due to loss of S1P,. Nonetheless, the idea that KLF2 also represses chemokine receptor gene expression is fascinating and further suggests that 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 this: what ultimately regulates KLF2 and emigration from the thymus? The prevention of autoimmunity by negative selection to tissue-specific Ags after positive selection is predicated on the retention of SP thymocytes in the medulla. KLF2 and S1P, are not highly expressed until at least 4 days after positive selection (6). So, what signals induce the up-regulation 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 (60). 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 responsiveness (61), which could explain the timing of KLF2 expression. However we found that T cells with conditional deficiency in ERK5 (62) had no defect in KLF2 expression or thymic emigration, and 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, naive T cells rapidly terminate KLF2 gene expression when activated by Ag (56, 63). Sinclair et al. recently demonstrated the PI3K signaling in T cells triggers KLF2 loss (64). 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, HSAlow thymocytes, consistent with a defect in emigration (64). Expression of the catalytic subunit of class IA PI3K by transgenic mice results in active PI3K signaling and a defect in emigration of thymocytes to the periphery, which may be due to decreased KLF2 expression (65). Likewise, activation of mature T cells directly in the thymus impairs their emigration (66), probably via a similar mechanism.
Conclusion
Although selection of T cells clones at the DP stage have been intensively studied, more recent research highlights the importance of postpositive 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 KL2, and the cell surface molecule S1P1, that are vital to this process and will be a focus for future research.

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References


18. We thank Tom McLaughtry and Steve Jameson for thoughtful discussion and critique.

19. Acknowledgments
20. References


