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T Cell Egress from the Thymus via Corticomedullary Blood Vessels

by

Marcus. A. Zachariah

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISO

This dissertation is dedicated to my parents, Abraham and Laila Zachariah, and my siblings, Marianne and Alyssa, for their love, guidance, and example.

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Contributions to the Presented Work

All of the work presented in this dissertation was performed under the direct supervision of Dr. Jason G. Cyster. Chapter 2 (excepting Figure 7 and the associated text) was published as Zachariah, M.A. and Cyster J.G., (2010). *Neural Crest-Derived Pericytes Promote Egress of Mature Thymocytes at the Corticomedullary Junction.* Science [Apr 22 Epub ahead of print]. Jason Cyster and I together designed the experiments presented. All experiments were performed by me, with Trung Pham providing help with experiments on Lyve1-Cre Sphk deficient mice (Figure 9) and Alex Bankovich providing help with experiments on Gai3 knockout chimeras (Figure 7). Ying Xu provided assistance generating S1P1 transgenic mice, and Jinping An helped generously with screening and maintaining mouse colonies. Other individuals who provided reagents and support are listed in the acknowledgments for Chapter 2.

T Cell Egress from the Thymus via

Corticomedullary Blood Vessels

by

Marcus A. Zachariah

While effective adaptive immunity depends on the migration of T cells out of the thymus, the requirements for and sites of thymocyte egress remain incompletely understood. Here, using an intravascular procedure to label emigrating cells, we find that mature single-positive thymocytes predominantly exit via blood vessels at the corticomedullary junction. Sphingosine-1-phosphate receptor-1 (S1P1) transgenic mice demonstrate that S1P1 is the only Kruppel-like factor-2 target necessary for egress. Intravascular labeling experiments aid the identification of roles for Gai3 and CD69 in negatively regulating egress. Finally, we reveal that neural-crest derived pericytes contribute to the sphingosine-1-phosphate that promotes thymic egress. These findings define the major thymic egress route, identify two thymic egress regulators and suggest a novel role for pericytes in promoting reverse transmigration of cells across endothelium.

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CHAPTER 1

Introduction

Human immunocompetence and survival depend on the egress of newly produced T cells from the thymus. Approximately 1% of the thymocyte population emigrates from the thymus each day to populate the peripheral T-cell compartment (*1, 2*). Following the arrival of early thymic progenitors, the thymus supports all steps of T-cell development, from the differentiation of progenitors into T-cell receptor (TCR)-expressing CD4 and CD8 double-positive (DP) cells in the cortex to the maturation of DP into CD4 or CD8 single-positive (SP) cells that localize to medullary regions. SP thymocytes initially possess an immature phenotype but over time acquire markers associated with maturation and subsequently egress from the thymus(*3, 4*).

Classic studies employed early thymectomy, models of congenital athymia, and intrathymic labeling to establish that the vast majority of T cells present in secondary lymphoid organs arise from the thymus (*5, 6*). Subsequent investigations sought to identify the route by which T cells emigrate and the mechanism by which egress occurs. The route of emigration could take place via blood vessels or lympatics, and there is data suggesting that both may occur (*7, 8*).

Electron microscopy revealed lymphocytes transmigrating across blood vessel endothelium (*9*) (*10*), lymphatic endothelium (*11*), or both sites (*12, 13*). Cannulation of vessels leaving the thymus and phenotypic evaluation of the lymphocytes within supported veins (*7*) or both veins and lymphatics (*14*) as the route of thymocyte egress. Due to the non-quantitative nature of many of these studies and the conflicting conclusions, the route that predominated remained unclear.

In work evaluating the mechanism of thymocyte emigration, Chaffin and Perlmutter generated transgenic mice expressing the S1 subunit of pertussis toxin under

control of the proximal promoter of the *lck* gene (*15, 16*). Thymocytes in these mice expressed the catalytic subunit of pertussis toxin, which inactivates Gi-mediated signaling by ADP-ribosylating a specific cysteine residue in the alpha subunit of Gi proteins. Pertussis toxin blocked emigration of thymocytes to the periphery, implicating a Gi-dependent process. Additional investigation suggested that thymocyte emigration may be sensitive to cytochalasin D and clostridium difficile toxin (*17*).

The discovery that thymocyte emigration was pertussis-sensitive sparked a series of efforts aimed at identifying the responsible Gi protein-coupled receptor(s). CXCR4 was the first receptor evaluated for a role in thymocyte egress. This receptor and its ligand SDF-1 were implicated in emigration from the thymus in a fetal thymic organ culture model(*18*). However, this combination of GPCR and ligand did not demonstrate an effect on thymic egress in several in vivo systems (*19, 20*). The next GPCR evaluated for a role in egress was CCR7. Egress of thymocytes from fetal thymus organ culture and from the neonatal thymus in vivo depended on CCR7 acting via the ligand CCL19 but not via the ligand CCL21. Surprisingly, CCR7 and its ligands did not measurably contribute to thymic egress in adult mice (*21, 22*). As such, the Gi protein-coupled receptor responsible for egress in adult mice remained unidentified.

The activity of THI and FTY720, two small molecules that blocked thymic egress, kindled the next series of advances (*23, 24*). Work by Mandala et al. revealed that a phosphoryl metabolite of FTY720 was an agonist for 4 of the 5 known receptors for the lysophospholipid S1P (*25*). Next, Matloubian et al. demonstrated that the particular S1P receptor responsible for the block in thymic egress caused by FTY720 was S1P1(*26*). Remarkably, genetic deletion of S1P1 phenocopied the lck-pertussis transgenic mouse,

indicating that S1P1 was the primary Gi-coupled receptor mediating egress. Subsequent work demonstrated that THI blocked egress by inhibiting the lysase responsible for gradients of S1P, which is normally low in the thymus and high in the blood and lymph (*27*). Eliminating S1P gradients by eliminating generation of S1P (*28*) also blocked thymic egress.

Mature CD62L-high single-positive thymocytes expressed S1P1 mRNA and migrated to S1P in vitro whereas immature (also called semimature) CD62L-low single positive thymocytes did not(*26*). This led to the following question: how is S1P1 transcription turned on only in mature thymocytes? Genetic deletion of KLF2 (*29, 30*), a zink-finger transcription factor, blocked thymic egress by inhibiting upregulation of S1P1 mRNA in mature thymocytes(*29*). KLF2 coimmunoprecipitated with the proximal S1P1 promoter and could transactivate the promoter. These data convincingly demonstrated that KLF2 controls transcription of S1P1 mRNA in mature thymocytes.

After the discovery of the importance of KLF2, several fundamental questions lept forward: what controls the upregulation of KLF2? Is this process somehow coupled to the completion of positive and negative selection? How is the timing of this upregulation determined? These questions are only beginning to be answered, but members of the Foxo family of transcription factors may be involved(*31, 32*).

While the mechanisms controlling the timing of KLF2 upregulation remain a mystery, recent advances have provided insight into the amount of time required for cells to become egress-competent. Building upon a strategy pioneered by Pam Fink's group(*33*), McCaughtry et al. employed Rag2p-GFP knockin mice to demonstrate that thymocyte medullary dwell time is four to five days rather than the two weeks duration

suggested by previous studies(*34*). More that 10% of CD4 single-positive thymocytes were negative for Rag2p-GFP, meaning that they had divided extensively, been subject to prolonged retention in the thymus, or reentered the thymus from the periphery. This GFP-negative subpopulation of cells would have confounded previous assessments of medullary dwell time.

To obtain a more accurate evaluation of the dwell time, the authors determined the half-life of Rag2p-GFP. Next, they compared Rag2p-GFP levels between the least mature CD4 single-positive thymocytes and recent thymic emigrants in the periphery, showing that medullary dwell time is four to five days - much shorter than previously thought. The authors also demonstrated that the CD4 single-positive thymocytes lowest in GFP possessed the surface-protein and mRNA expression phenotype of egress-competent cells. This indicates that thymocyte egress occurs in a fashion similar to a conveyor belt - cells that enter the medulla first exit the thymus first.

Based on the knowledge that egress-competent thymocytes reside in the medulla, one might imagine that sites capable of permitting egress would be restricted to this region. However, data from mice deficient in CCR7 or its ligands suggested otherwise (*22, 35*). CCR7 deficient thymocytes were deficient in their ability to migrate from cortex to medulla, yet they egressed normally. FTY720-mediated egress block in CCR7-deficient mice led to the accumulation of cells with an egress-comptetent phenotype in the cortex. These data argue that sites through which egress can occur exist in both thymic cortex and medulla.

Potential routes of egress from the thymus include veins and lymphatics. Like the epidermis, the thymic epithelial space does not contain blood vessels or

lymphatics (*13*). Thus egress via either veins or lymphatics would require traversal of the thymic perivascular space (*36*). Several recent papers have provided advances into the localization of lymphatics in the thymus (*37, 38*), the phenotype of blood vessels in the thymus (*39-42*), and the surprising origin of cells that line the perivascular space (*43-45*).

Identifying the cells that generate the S1P required for egress may provide insight into the egress route. When the two sphingosine kinases required for generating S1P were deleted from interferon-responsive cells using a Mx1-Cre transgenic mouse, thymic egress was blocked(*28*). Mature thymocytes accumulated 4-fold over the number present in control mice. This suggests that the cells generating the S1P required for thymic egress are interferon-responsive. Among interferon-responsive cells, the radiation-resistant and radiation-sensitive compartments both contribute to thymic egress, but surprisingly the radiation-resistant source of S1P holds more importance. When lethally-irradiated wild-type mice were reconstituted with sphingosine kinase-deficient bone marrow, mature thymocytes did not accumulate. In reciprocal chimeras (sphingosine kinase-deficient mice reconstituted with wild-type bone marrow), thymocytes accumulated 50% above the number in control mice. These results argue that the primary source of S1P required for thymic egress is interferon-responsive and radiation-resistant.

Irradiated kinase-deficient mice reconstituted with wild-type bone marrow possess normal plasma S1P but reduced lymph S1P. The partial restoration of thymocyte egress in these mice would seem to suggest that thymocyte egress can occur into the blood. This would be consistent with the subtle restoration of thymocyte egress in kinase deficient mice transfused with wild type RBC to restore plasma S1P levels to normal. However, these data can only be taken to indicate that thymocyte egress can occur into

the blood when egress across lymphatic endothelium is blocked. Moreover, in both these systems lymph S1P remains low at the cisternae chylae, but lymph S1P in thymic lymphatics may be higher due to the unique origin of these lymphatics in the perivascular space adjacent to thin-walled blood vessels(*11*). Direct imaging of the egress process, may be necessary to identify the predominant route of egress.

Whether into lymphatics or blood vessels, thymic egress must involve reverse transmigration across an endothelial barrier. Several recent observations suggest that molecules required for reorganization of the thymocyte cytoskeleton are central to this process. Coronin 1a (also known as coronin 1, coro1a, TACO, or p57) belongs to a family of actin regulators present in all eukaryotes(*46*). In the T cell lineage, coronin1a is most highly expressed in single-positive thymoyctes, hinting at a possible role in the migration of these cells(*47*). Recent work implicates coronin1a in thymocyte egress and peripheral homing(*48-51*), as well as T cell survival(*48, 49, 51, 52*).

Mice in which coronin 1a was genetically deleted possessed a reduced number of recent thymic emigrants as measured by fitc intrathymic injection(*48*). Interpretation of this result is complicated by defects in peripheral homing and T cell survival in these mice. However, additional data demonstrating an increase in the fractional representation (if not absolute number) of mature thymocytes (reproduced by ref. (*49*)) and a reduction of egress from an *in vitro* thymic organ culture model support the notion that coronin 1a-deficient mice suffer from a block in thymic egress. Inhibition of F-actin formation by coronin 1a might explain this egress block. Coronin 1a-deficient T cells possessed higher levels of steady state F-actin, a phenotype which could be rescued by wild-type coronin 1a but not two coronin 1a mutants compromised in their ability to bind Arp2/3.

Subsequently, Shiow et al. (*51*) provided evidence indicating that a mutation in coronin 1a could explain the defect in thymic egress associated with cataract Shionogi mice(*53, 54*). These mice contained a recessive point mutation in exon 2 of *Coro1a* that results in a glutamic acid to lysine substitution at residue 26 in the Beta propeller domain of the protein. The mutation enhanced coronin 1a's inhibition of Arp2/3 and resulted coronin 1a's mislocalization from the leading edge of migrating T cells. Homozygous mice possessed a numerical accumulation of mature single-positive thymocytes, a loss of peripheral T cells, and defects in peripheral trafficking. Namely, entry into and egress from lymph nodes was reduced, and T cells within the lymph node moved slower and with less directionality when observed by two-photon microscopy. Additionally, a coronin 1a hypomorph was identified in a murine forward genetic screen for peripheral T cell deficiencies. This prompted the evaluation of 16 human patients with T-B+NK+ SCID of unknown etiology for mutations in coronin 1a, and one patient was demonstrated to have mutations in both CORO1A alleles(*50*).

Aside from coronin 1a, several additional molecules involved in cytoskeletal reorganization may play critical roles during thymocyte egress. Genetic deletion of DOCK2, a hematoipoietic cell-specific CDM family protein that funcitons upstream of Rac, resulted in peripheral Tcell deficiency(*55*). The authors observed impaired homing to secondary lympoid organs in vivo and defective thymocyte egress measured by an in vitro model. Evaluation of DOCK2's effect on thymocyte egress in vivo will be important to confirm in the vitro data.

The phenotype of knockout mice deficient in mDIA suggested a role for this actin-nucleating protein in thymic egress(*56*). The knockout mice exhibited a loss of T

cells but not B cells, CD11c+ cells, or CD11b+ cells from secondary lymphoid organs. A numerical accumulation of mature (CD69-low CD62L-high) thymocytes accompanied the peripheral T cell deficiency. Additionally, the authors showed reduced emigration of mDIA-deficient cells from a thymic organ culture system. These results strongly support the importance of mDIA in egress from the thymus.

In addition to molecules involved in cytoskeletal reorganization, cell-to-cell adhesion molecules may play a role in thymocyte egress. Several known forms of lymphocyte transmigration, including entry into the thymus(*41*), lymph nodes (*57*), and brain(*58, 59*), require the combined action of a Gi-coupled protein receptor and cell-to-cell adhesion molecules. Recent data indicates that PSGL-1, the only known ligand of P-selectin, may be involved in egress from the thymus. P-selectin marks a subset of thymic endothelial cells, and thymocytes express PSGL-1(*41, 60*). PSGL-1-deficient mice had an increased frequency of immature (also called semimature) and mature single-positive thymocytes(*61*). This finding, combined with a twofold decrease in circulating T cells in the periphery, suggested a possible thymic egress defect. The accumulation of thymocytes at the immature single-positve stage is unusual for a thymic egress defect, as other strains of mice with egress defects selectively accumulate mature single-positive cells(*51, 62*). However, two additional approaches (fitc intrathymic injection and T cell receptor excision analysis) supported the authors' conclusion that egress is affected.

The work presented here aims to address several questions. First, is S1P1 upregulation sufficient to account for the acquisition of egress competence by mature single-positive thymocytes, or are other changes required?

Second, what molecules are involved in retaining cells in the thymus?

Interestingly, elimination of Gi protein-coupled receptor signaling with pertussis toxin promoted egress in the periphery (*63*) and bone marrow (*64*) but caused retention in the thymus (*15, 16*). Thymocytes express both Gai2 and Gai3. Genetic deletion of Gai2 apparently did not affect egress (*65*) – does Gai3 play a role?

Is CD69 involved in thymic retention? Specific agonism of S1P1 by SEW287 rapidly downmodulated CD69 in the thymus (*66*), and CD69 could retain cells in the thymus when overexpressed to non-physiological levels in transgenic mice (*67, 68*). However, CD69 knockout mice called into question the role of CD69 in thymic retention. While knockout mice revealed an essential role for CD69 in peripheral retention (*51*), the knockout did not demonstrate any phenotype in the thymus (*69*). Do CD69 knockout mice possess any hitherto unobserved thymic phenotype? If not, what explains the different roles for this protein in the thymus and the periphery?

Third, where does egress occur? Does it occur predominantly into blood vessels or lymphatics? Can we visualize this migration process directly?

Fourth, what are the cells types that produced the S1P required for thymocyte egress? Do they include blood vessel endothelium, lymphatic endothelium, or some unexpected type of cell? Existing data highlights the primacy of an interferon-responsive, radioresistant, and probably local source (*28*).

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CHAPTER 2

Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction

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Summary

While effective adaptive immunity depends on the migration of T cells out of the thymus, the requirements for and sites of thymocyte egress remain incompletely understood. Here, using an intravascular procedure to label emigrating cells, we find that mature single-positive thymocytes predominantly exit via blood vessels at the corticomedullary junction. Sphingosine-1-phosphate receptor-1 (S1P1) transgenic mice demonstrate that S1P1 is the only Kruppel-like factor-2 target necessary for egress. Intravascular labeling experiments aid the identification of roles for Gai3 and CD69 in negatively regulating egress. Finally, we reveal that neural-crest derived pericytes contribute to the sphingosine-1-phosphate that promotes thymic egress. These findings define the major thymic egress route, identify two thymic egress regulators and suggest a novel role for pericytes in promoting reverse transmigration of cells across endothelium.

Introduction

The thymus is an essential site of T cell development and tolerance induction. CD4 and CD8 double negative (DN) precursors develop into double positive (DP) thymocytes in the thymic cortex, and positively selected cells give rise to semi-mature single positive (SP) thymocytes that undergo negative selection and maturation in the medulla. Over a period of a few days, SP thymocytes upregulate Kruppel-like factor (KLF) 2 and KLF2 target genes, including sphingosine-1-phosphate receptor-1 (S1P1), and exit the thymus in an S1P1 and sphingosine-1-phosphate (S1P)-dependent manner (*1, 2*). The route by which egress occurs has not yet been identified. Electron microscopy studies have occasionally (non-quantitatively) identified cells crossing blood vessels at the corticomedullary junction (*3-6*). However, thymic progenitor cells are thought to enter the thymus at this location (*7, 8*) and it was not possible in these studies to determine whether the transmigrating cells were entering or exiting the thymus. Adding to the complexity, a number of studies have suggested a role for lymphatics in thymic egress (*6, 9-12*), though other studies have emphasized the rarity of lymphatics in the thymus (*3*).

S1P1 is a Gai coupled receptor and early studies showed that inhibition of Gai by transgenic expression of pertussis toxin (PTX) A subunit was sufficient to inhibit thymic egress (*13*). The major PTX sensitive Gai-family proteins expressed in T cells are Gai2 and Gai3 (*14-16*). In mice lacking Gai2, thymopoiesis is altered, apparently due to a role for this G-protein in thymocyte maturation rather than an essential role in thymic egress (*17*). Whether Gai3 has a role in thymic egress has not been determined.

Semi-mature SP thymocytes express high amounts of CD69 and heat stable antigen (HSA, also known as CD24) and low levels of Qa2 and CD62L. These markers reverse in expression during final maturation (*18, 19*). CD69 is down-regulated on thymocytes coincident with S1P1 upregulation (*20*). In the periphery, CD69 upregulation on activated T cells negatively regulates S1P1 function and inhibits T cell egress from lymph nodes (*21*). Although transgenic overexpression of CD69 in the thymus caused thymocyte retention (*22, 23*), CD69 knockout mice were not reported to have a thymic phenotype (*24, 25*) and the physiological role of CD69 expression by thymocytes has been uncertain.

S1P is generated through the action of two sphingosine kinases (Sphks) and can be made intracellulary by all cell types as part of their sphingolipid metabolism program (*26*). However, only a limited number of cell types secrete S1P (*27*). In mice lacking Sphk-2 in all cells and Sphk-1 in type I IFN responsive cells, thymic egress is impaired (*28*). This study showed that the major S1P source required for egress was radiation resistant. Based on in vitro observations it has been speculated that a key radiation resistant S1P source is endothelial cells (*29*).

Thymic egress uniquely involves reverse (basolateral-to-apical) transmigration of T cells across blood vessels. Prior to engaging the endothelial cell, egressing thymocytes are likely to make contact with pericytes, the specialized support cells that surround blood vessels (*30*). Recent studies established that thymic pericytes are of neural crest origin (*31, 32*). The role of pericytes, if any, in leukocyte transmigration across blood vessels has not been well defined.

Here, using a transgenic approach, we establish that S1P1 is sufficient to promote egress of KLF2-negative DP cells and KLF2$^{-/-}$ mature SP thymocytes, indicating that S1P1 is the key KLF2-induced gene needed for egress. The elevated frequency of egressing cells in S1P1 transgenic mice facilitated development of a method to *in vivo* label emigrating CD4 T cells, and by this approach we establish that egress normally occurs via vessels at the corticomedullary junction. We also identify roles for Gai3 and CD69 in negatively regulating thymic egress. Finally, utilizing Wnt1-Cre mice to achieve Sphk ablation in neural crest-derived pericytes, we identify these cells as a critical source of S1P required for normal T cell reverse transmigration across thymic vessels.

Results

S1P1 is sufficient to mediate thymic egress

By inducing expression of the pertussis toxin A subunit in thymocytes using Lck-Cre, we found that inhibition of Gai caused a similar extent of mature SP thymocyte accumulation (Fig. 1a) to that caused by S1P1-deficiency (*20*). To examine whether S1P1 upregulation is the sole maturation event occurring in SP thymocytes that is necessary for their egress, we asked whether premature expression of S1P1 on immature thymocytes was sufficient to promote egress. Transgenic mice carrying an S1P1 transgene under control of the Lck proximal promoter and Ig heavy chain enhancer (*33*) were generated and screened for S1P1 expression in thymocytes. One line (line A) showed abundant expression in DP thymocytes and semi-mature SP thymocytes (Fig. 1a) and a second line (line D) had lower but detectable expression in these cells (Fig. 2a). In chemotaxis assays S1P1A transgenic DP and semi-mature SP thymocytes showed strong migratory responses to S1P (Fig. 2b). Analysis of blood and spleen samples from these mice revealed the presence of substantial numbers of DP thymocytes (Fig. 2c) and there was a reduction of these cells within the thymus (Fig. 2c). To determine whether there was premature egress of SP thymocytes we intercrossed S1P1A transgenic mice with RAG-GFP reporter mice (*34*). Enumeration of CD4 T cells in the periphery that had the RAG-GFP^{hi}Qa2^{int} phenotype of recent thymic emigrants (*19*) revealed they were present in elevated frequencies and numbers in the transgenic mice (Fig. 2d). These findings indicate that S1P1 is sufficient to promote egress of DP thymocytes, cells that lack KLF2 (*35*), as well as facilitating egress of less mature SP thymoctyes. To directly test whether

S1P1 was the sole KLF2-target gene needed for egress of SP thymocytes, we intercrossed S1P1A transgenic and KLF2$^{f/f}$-CD4Cre mice (*36*) and enumerated mature S1P1^{+} cells in the thymus. Since the S1P1 transgene is under the control of the Lck promoter and Eμ enhancer it was not expected to be KLF2 regulated and no difference in S1P1 staining was detected between KLF2$^{f/f}$-CD4Cre S1P1A transgenic and wildtype S1P1A transgenic SP thymocytes (Fig. 2e). In contrast to findings in KLF2-deficient non-transgenic mice, where mature SP thymocytes accumulate (Fig. 2e and (*35*)), there was no accumulation of mature HSA^{lo}Qa2^{hi} SP thymocytes in KLF2-deficient S1P1 transgenic mice compared to control S1P1 transgenic mice (Fig. 2e) indicating that egress of the transgenic SP cells was not KLF2 dependent. These observations suggest that S1P1 is the only KLF2 target gene essential for thymocyte emigration.

In sections of S1P1A and S1P1D transgenic thymi, unusual perivascular accumulations of thymocytes were observed, most strikingly in the cortex but also detectable in the medulla (Fig. 2f). In the cortex, transgenic cells accumulated between the ERTR7^{+} mesenchymal cells associated with CK5^{+} epithelial cells and the basement membrane of the blood vessels and associated ERTR7^{+} pericytes (Fig. 2f). Treatment with FTY720 to modulate thymocyte S1P1 function caused a reduction in the perivascular accumulations suggesting they were maintained by ongoing S1P responses (Fig. 2f). These observations suggest that there is local availability of S1P in the immediate vicinity of thymic blood vessels.

To explore the possibility that premature egress might be associated with diminished negative selection and autoimmunity, various organs were examined for cellular infiltrates (Fig. 3). Large lymphoid infiltrates were observed in salivary gland,

lacrimal gland and lung at a significantly higher frequency in the S1P1A transgenic mice than in littermate control mice (Fig. 3). Recent findings have shown elevated S1P1 expression diminishes Treg development (*37*) and we also observed reduced Treg numbers in the thymi of S1P1A transgenic mice (Fig. 3). However, peripheral Treg frequencies were not affected (Fig. 3) and previous studies have shown that even small numbers of Treg can protect from disease (*38*). Thus, while we cannot exclude an effect of the S1P1A transgene on peripheral Treg function, our results are consistent with the possibility that premature thymic egress of developing T cells is associated with insufficient negative selection and autoimmunity.

***In vivo* labeling identifies sites of thymocyte egress**

The heightened frequency of emigrating thymocytes in S1P1A transgenic mice led us to ask if we could detect thymocytes in the act of egress. Previous studies have shown that very short treatments with labeled antibodies, particularly if conjugated to the large fluorophore phycoerythrin (PE), achieve selective labeling of cells exposed to the vascular compartment (*39*). Mice were injected intravenously with PE-conjugated CD4 antibody (CD4PE) and a few minutes later the thymus was isolated and prepared for flow cytometric analysis and immunofluorescence microscopy. In S1P1A transgenic mice we observed a low but reproducible frequency of DP thymocytes that became CD4PE-labeled (Fig. 2a). No DP cells became labeled in nontransgenic littermate controls (Fig. 4a). The labeled DP cells in transgenic mice did not simply reflect blood contamination because the frequency of DP cells amongst the CD4PE-labeled cells was highly enriched compared to their frequency amongst CD4PE-labeled cells in venous blood (Fig. 4a).

When DP cells were transferred directly into the blood they disappeared with a half-life of about 3 hours (Fig. 5), suggesting they are lost rapidly once they leave the thymus and enter general circulation. In sections, the S1P1A transgenic cells labeled following intravenous CD4PE antibody treatment were located in blood vessels distributed through both the cortex and medulla (Fig. 4b and 6). In some cases the CD4PE labeled cells were found to be spanning the $CD31^+$ endothelium, apparently caught in the act of crossing (Fig. 4b).

By collecting large (~5 million event) flow cytometric files it was possible to observe intravascularly CD4PE labeled SP thymocytes in normal mice (Fig. 4a and c). Using RAG-GFP reporter mice to track thymocyte maturity (*19*), the majority (~80%) of intravascularly CD4PE labeled cells in the thymus had higher amounts of GFP than cells in the blood, indicating that these were cells that had just emigrated but not yet left the thymus, rather than corresponding to cells already present in blood circulation (Fig. 4d). These thymic cells also expressed higher amounts of CD31 than cells in the blood or, interestingly, the most mature cells in the thymus (Fig. 4d) suggesting they had transiently acquired or upregulated CD31 during egress.

Immunofluorescence analysis of sections from *in vivo* labeled wild-type thymus revealed rare PE-labeled cells in vessels near the corticomedullary junction (Fig. 4e-h). Enumeration of CD4PE-labeled cells across a 30 μm thick thymic cross-section revealed a mean of 70 cells per section (Fig. 4f and Fig 6a) with the great majority being located in vessels within 50 μm of the corticomedullary junction (Fig. 4e and f). Using the number

of cells present in the volume of one section to extrapolate the number present in the volume of the whole organ (~0.025 cubic centimeters), we calculated that roughly 2500 labeled cells are present per thymus. This number agrees closely with that obtained by flow cytometry (Fig. 4c). If we assume that thymocyte reverse transmigration takes approximately 3 minutes, then we estimate 1.2 million cells exit the thymus per day. This number is in close agreement with the estimate that 1% of total thymocytes (~1 million cells) egress per day (*40*). Close examination of *in vivo* labeled thymic sections revealed examples of cells that appeared to be in the act of reverse transmigrating across the vascular endothelium into the blood (Fig. 5g and h and Fig. 6b and c). Analysis of serial sections confirmed that the cells were projecting through the $CD31^+$ endothelium (Suppl. Movie 1 and 2). In summary, we establish a method to identify cells exiting the thymus and demonstrate that the majority of thymocytes leave by blood vessels at the corticomedullary junction.

Gai3 negatively regulates S1P1 and thymic egress

Although the impact of Gai2-deficiency on thymic development and egress has been examined (*17*) the role of Gai3 has not been determined. Mature SP thymocytes express Gai2 and Gai3 while expressing little Gai1 (http://www.immgen.org/index_content.html). Analysis of thymi from Gai3-deficient BM chimeras revealed normal numbers of DP and semi-mature SP thymocytes but an underrepresentation of mature $HSA^{lo}CD62L^{hi}$ SP cells (Fig. 7a). In the periphery, increased numbers of $HSA^{int}Qa2^{lo}$ recent thymic emigrants were detected (Fig. 7b). Intravascular CD4PE labeling revealed that emigrating thymocytes were enriched for $Qa2^{lo}HSA^{int}$ cells (Fig. 7c). S1P1 expression was elevated

on mature SP cells within the thymus (Fig. 7d) and the cells exhibited augmented chemotaxis to S1P but not CCL21 or CXCL12 (Fig. 7e). These findings suggest that Gai3 normally causes a reduction in surface S1P1 expression in developing thymocytes, reducing S1P responsiveness and delaying egress.

CD69 retards thymic egress

CD69 is upregulated on thymocytes during the DP to SP transition, being expressed at high amounts on semi-mature SP cells and present in lower amounts on a fraction of the mature SP cells (*18, 20*). Although CD69-deficiency was not reported to alter the total number of SP cells in the thymus (*24, 25*), it seemed possible that removal of this S1P1-negative regulator might cause a measurable enhancement in egress rate and we therefore intercrossed CD69-deficient mice with RAG-GFP reporter mice to examine the maturation state of newly emigrating thymocytes. CD69-deficient mice had a decrease in the frequency of mature SP cells in the thymus (Fig. 8a) and an increase in $GFP^{hi}Qa2^{int}$ cells in the periphery (Fig. 8b). Based on previous measurements of GFP half-life (*41*) and the difference in GFP level between intravascular CD4PE-labeled cells in CD69-deficient and control mice, we estimate that CD69-deficient cells exit the thymus about 10 hours faster than control cells (Fig. 8c). Analysis of CD94-deficient mice that also carry the 129-derived NK gene cluster but have wildtype CD69 ruled out the possibility that the altered thymic egress was due to polymorphism in another NK complex gene (Fig. 8a). In chemotaxis assays, CD69-deficient mature CD4 SP thymocytes showed a small but reproducible increase in S1P responsiveness (Fig. 8d). In summary, CD69 has a homeostatic role in delaying egress of maturing thymocytes.

Neural crest-derived pericytes promote thymic egress

S1P is present at high concentration in blood and low concentration in the thymus (*42*). Previous work established the major source of S1P necessary for thymic egress as radiation resistant (*43*). This suggested, surprisingly, that blood S1P is not sufficient to promote normal egress and S1P production by a local stromal cell type might be required.

The perivascular accumulations of S1P1 transgenic mice and recent evidence (*31, 32*) revealing the unexpected origin of thymic pericytes in the neural crest led us to hypothesize that S1P produced by this unique cell type might be required for egress. Wnt1-Cre, an established neural crest precursor-specific Cre (*44*) causes efficient recombination of the RosaYFP locus in the majority of thymic pericytes (*31, 32*). To test for a possible role of pericytes as a radiation resistant S1P source necessary for thymocyte egress, Wnt1-Cre mice were intercrossed with $Sphk1^{f/-}$ $Sphk2^{-/-}$ mice. Wnt1-Cre^{+} $Sphk1^{f/-}$ or $^{f/f}$ $Sphk2^{-/-}$ mice were viable and showed no gross abnormalities, suggesting intrinsic Sphk-activity may not be essential for neural crest function during development. However, analysis of Wnt1-Cre^{+} $Sphk1^{f/-}$ or $^{f/f}$ $Sphk2^{-/-}$ mice revealed a selective accumulation of mature SP thymocytes compared to littermate controls (Fig. 9a). This accumulation approached that observed in polyI:C treated MxCre Sphk-deficient mice (Fig. 9a). Intravenous CD4PE antibody treatment showed a reduced number of vascularly exposed CD4 SP thymocytes in the mice lacking Sphk activity in neural crest cell-derived pericytes (Fig. 9b) and there were reduced numbers of $HSA^{hi}Qa2^{int}$ recent thymic emigrants in peripheral LNs (Fig. 9c). Mature T cell numbers

in LNs, spleen and blood were in the normal range (Fig 9c and data not shown). S1P1 surface abundance was slightly elevated on mature SP thymocytes in these mice, consistent with reduced exposure to S1P (Fig. 9d). Despite the accumulation of mature SP thymocytes in Wnt1-Cre Sphk-deficient mice (Fig. 9a) there was a lower frequency of CD69-negative cells amongst this population compared to littermate controls (Fig. 9e). This may reflect a requirement for S1P exposure for full CD69 downregulation to occur. Immunohistochemical analysis established that the pericyte distribution in the thymus of Wnt1-Cre Sphk-deficient mice was normal (Fig. 9f). To determine whether the thymic egress block in polyI:C treated MxCre Sphk-deficient mice could also reflect gene deletion in pericytes, we examined RosaYFP reporter expression in isolated cells by flow cytometry (Fig. 9g). Pericytes, identified as $PDGFRb^{+}CD31^{-}CD45^{-}$ cells (*32*), showed near complete reporter gene activation whereas blood endothelial cells showed partial reporter gene activation (Fig. 9g). The findings from the Wnt1-Cre and MxCre Sphk-deficient mice define an unexpected and critical role for pericytes in producing S1P required for thymocyte egress.

Some studies have suggested a role for lymphatic vessels in thymocyte egress (*6, 9-12*). In our studies, lymphatic vessels were rarely detected in cross sections of adult C57Bl/6 mouse thymi (not shown) and LYVE1+ lymphatic endothelial cells were very rare by flow cytometric analysis of enzyme digested thymi, being outnumbered by blood vessel endothelium by at least 50 to 1 (Fig. 9g and 9h). To further test whether lymphatic endothelium played a role in thymic egress, we analyzed LYVE1-Cre^{+} $Sphk1^{f/f}Sphk2^{-/-}$ mice (*45*) . LYVE1-Cre causes efficient floxed gene ablation in lymphatic endothelial

cells and partial ablation in blood vasculature (*45*). Analysis of LYVE1-Cre x RosaYFP mice revealed YFP expression in ~40% of thymic blood vessel endothelial cells (Fig. 9h); there were too few lymphatic endothelial cells in the thymus to permit reporter analysis in these cells. However, in lymph nodes, lymphatic endothelial cells were successfully isolated (Fig. 9h) confirming the efficacy of our digestion protocol, and all the lymphatic cells were YFP reporter+ (Fig. 9h) as expected (*45*). In LYVE1-Cre^{+} Sphk-deficient mice there was no increase in mature SP thymocyte numbers in the thymus (Fig. 9i). Taken together, these findings establish a role for neural crest-derived pericytes as a source of S1P necessary for thymic egress and argue against a role for lymphatic vessels in thymic egress.

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Figure Legends

Figure 1. LckCre x RosaPTX mice show accumulation of mature SP thymocytes. (a) Number of mature SP thymocytes in LckCre x *Rosa26*^{PTX} and control (Cre-ve *Rosa26*^{PTX}) mice. (b-d) Number of CD4 and CD8 T cells in blood (b) spleen (c) and peripheral LNs (d). Bars indicate means and circles individual mice.

Figure 2. Transgenic S1P1 expression promotes egress of DP and KLF2-deficient SP thymocytes and causes their perivascular accumulation. (a) Flow cytometric analysis of S1P1 expression by DP, semi-mature and mature SP thymocytes in S1P1A and S1P1D transgenic and control (-) mice. (b) Transwell migration of S1P1A transgenic and control DP and semi-mature SP thymocytes to S1P, CXCL12 (0.3 μg/ml) and CCL21 (1.0 μg/ml). (c) Flow cytometric detection of DP thymocytes in blood of S1P1A transgenic mice. Bar graph shows numbers of cells in the blood, spleen and thymus of S1P1A and S1P1D transgenic and control mice with bars showing means and circles values for individual mice. (d) Flow cytometric detection of semi-mature RAG-GFP^{hi}Qa2^{int} CD4 T cells in blood of S1P1A and control RAG-GFP mice. Graphs on right show enumeration of these cells in blood and spleen. (e) Number of (lower graphs) and S1P1 expression by (upper plots) mature CD62L^{hi}Qa2^{int} CD4 SP thymocytes in KLF2$^{f/+}$ or KLF2$^{f/f}$ CD4Cre^{+} control (-) and S1P1A transgenic mice. (f) Immunohistochemical analysis of thymus sections from S1P1 transgenic and control mice showing accumulation of transgenic thymocytes in perivascular regions and the reduction following 24 h FTY720 treatment. Sections were stained with antibodies to detect the indicated markers. Scale bars represent 200 μm (left column) or 10 μm (middle and right columns).

Figure 3. Cellular infiltrates in multiple organs of S1P1A-transgenic mice and thymic Treg frequency. (a) Reduced frequency of CD3^{hi}CCR7^{hi} DP thymocytes in

S1P1A-transgenic thymus and enrichment for these cells amongst DP thymocytes in the spleen. (b) Transwell migration of S1P1A-transgenic total DP and $CD3^{hi}CCR7^{hi}$ DP thymocytes to S1P, CXCL12 (0.3 μg/ml) and CCL21 (1.0 μg/ml). Circles in (a) indicate individual mice whereas circles in (b) indicate duplicate transwells for a single mouse. Data represent at least 5 mice from 3 experiments in (a) and are representative of two mice analyzed from two experiments in (b). (c) Haematoxylin and eosin staining of the indicated fixed tissues (left panels) showing examples of infiltrates detected in the S1P1A-transgenic. Scale bar indicates 100 μm. Right plots show summary of data for individual mice where a filled box indicates the presence of an infiltrate in the tissue type indicated by the lower panel. (d) Flow cytometric analysis of CD4 SP thymocytes showing frequency of $FoxP3^{+}CD25^{+}$ cells. Bar graphs show frequency of $FoxP3^{+}CD25^{+}$ cells amongst CD4 SP thymocytes, splenocytes or brachial lymph node cells from control (-) and S1P1A-transgenic (A) mice. Points indicate individual mice and bars represent means. Five mice of each type were analyzed in three experiments.

Figure 4. *In vivo* labeling identifies emigrating CD4 SP thymocytes. (a) Flow cytometric detection of intravascularly CD4PE labeled DP cells in the thymus of S1P1A transgenic versus control (-) mice. Thymocytes were isolated 5 minutes after CD4PE antibody injection. Bar graph shows percent of CD4PE-positive cells that were also CD8-positive, summarizing data from 3 mice with bars showing mean and circles individual mice. (b) Immunofluorescence of thymic section from an S1P1A transgenic pretreated with CD4PE. Panels 1-10 show a confocal z-series through an emigrating DP cell (0.3um z step). Single color panel shows CD8 staining, indicating cortical location of transmigration. Scale bars represent 10 (top) or 5 (bottom) μm. (c) Flow cytometric detection of intravascularly CD4PE labeled SP cells in the thymus of non-transgenic mice and their absence following FTY720 treatment. Bars in right graph show mean of 3

experiments. (d) RAG-GFP intensity and CD31 staining on the indicated CD4PE labeled cells. Top left histogram plot shows GFP fluorescence in intravascularly CD4PE labeled cells in thymus, mature $CD62L^{hi}Qa2^{int}$ CD4 SP thymocytes, and CD4 T cells in blood and numbers indicate % of GFP^{hi} cells. Top right graph shows summarized data for 3 mice. Bottom histogram plot shows CD31 staining with inset fluorescent intensities for the indicated thymic populations and RAG-GFP^{hi} blood recent thymic emigrants (RTE). (e-h) Immunofluorescence of thymic sections from non-transgenic mice injected with anti-CD4PE, also stained to detect vascular endothelium with anti-CD31 (green) and with CD8 (e) or DAPI (g-h) to detect nuclei (blue). (e) Tiled view of an entire thymic section with CD4PE+ cells highlighted by red squares and an example shown in the inset. Scale bars indicate 25 μm (inset image) and 800 μm (main image). (f) Enumeration of CD4PE labeled cells in thymic cross sections, assigned as being located in vessels within 50 μm of the corticomedullary junction, in the cortex or in the medulla. (g, h) High power views of transmigrating thymocytes. Large left panel displays a single 0.24 μm thick optical section (xy). Single color panels: upper left, CD4; upper right, DAPI; lower left, CD31; lower right, 3 color overlay. Single panels are 0.1 μm thick xz optical sections in (g) and 0.24 μm thick xy optical sections in (h). Diagrams on right of each set of panels included for clarity. Scale bars indicate 10 μm for large left panel and 1 (g) or 5 (h) μm for small right panels.

Figure 5. Rapid disappearance of DP thymocytes from the periphery and immunofluorescence analysis of IV $CD4PE^{+}$ cell distribution in S1P1A-transgenic thymus. (a) Number of DP cells in blood and spleen at indicated time points after intravenous thymocyte transfer. (b and c) Immunofluorescence of thymic section from S1P1A-transgenic mouse injected with anti-CD4PE, stained to detect vascular endothelium

with anti-CD31 (green) and for cortical regions with anti-CD8 (blue). b and c are example regions from the tiled view of a thymic lobe. Data are representative of at least two mice analyzed in two experiments.

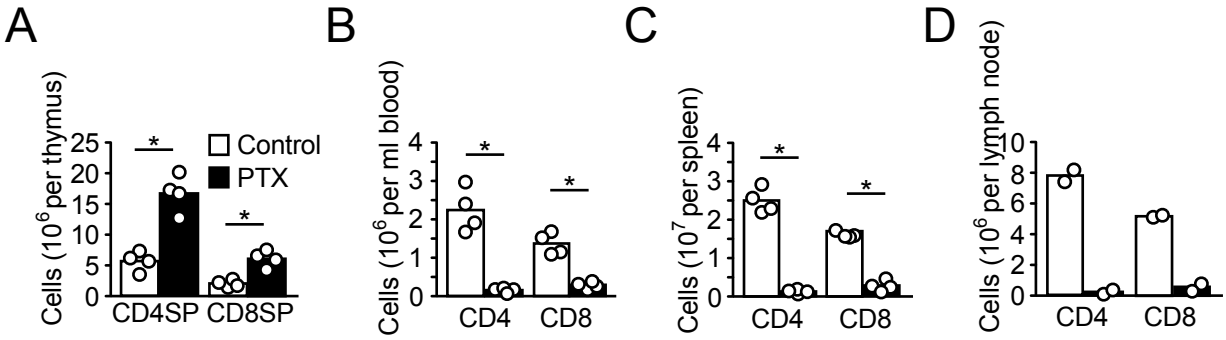
Figure 6. Immunofluorescence analysis of IV CD4PE$_{+}$ cell distribution in the thymus. (a-c) Immunofluorescence of thymic sections from non-transgenic mice injected with anti-CD4PE, also stained to detect vascular endothelium with anti-CD31 (green) and with either the medullary cytokeratin marker CK5 (blue, a) or with DAPI to detect nuclei (blue, b and c). Section in (a) is serial to the section in Figure 4A. Scale bars indicate 5 μm (inset image) and 800 μm (main image) in (a), 20 μm (left) and 5 μm (right) in (b), and 10 μm (left) and 5 μm (right) in (c). Data are representative of thymic sections from at least three mice.

Figure 7. Gai3 deficient thymocytes egress prematurely and have elevated S1P1 surface expression. (a) Flow cytometric analysis of thymocytes from control and Gai3-deficient bone marrow chimeras showing CD4 and CD8 staining of total thymocytes (left panels) and HSA and CD62L staining of CD4 SP thymocytes (center panels). Numbers indicate cell frequencies in the indicated gates. Bars in right graph show mean and circles data for individual mice. Semi 4 indicates HSA^{hi}CD62L^{lo} cells and Mat 4 indicates HSA^{lo}CD62L^{hi} cells, as gated in the center panels. (b) Flow cytometric detection (left panels) and number (right panel) of WT and Gai3-deficient HSA^{hi}Qa2^{int} recent thymic emigrants (RTE) in peripheral LNs. (c) Flow cytometric detection of HSA and Qa2 on intravascularly CD4PE-labeled thymocytes. (d) Flow cytometric analysis (left panel) and summary mean fluorescent intensity data (right panel) of S1P1 expression by HSA^{lo}Qa2^{hi} mature SP thymocytes from Gai3-deficient and control BM chimeras. (e) Transwell migration assay of Gai3-deficient and control mature CD4 SP thymocytes to S1P, CCL21 (1 μg/ml) and CXCL12 (0.3 μg/ml).

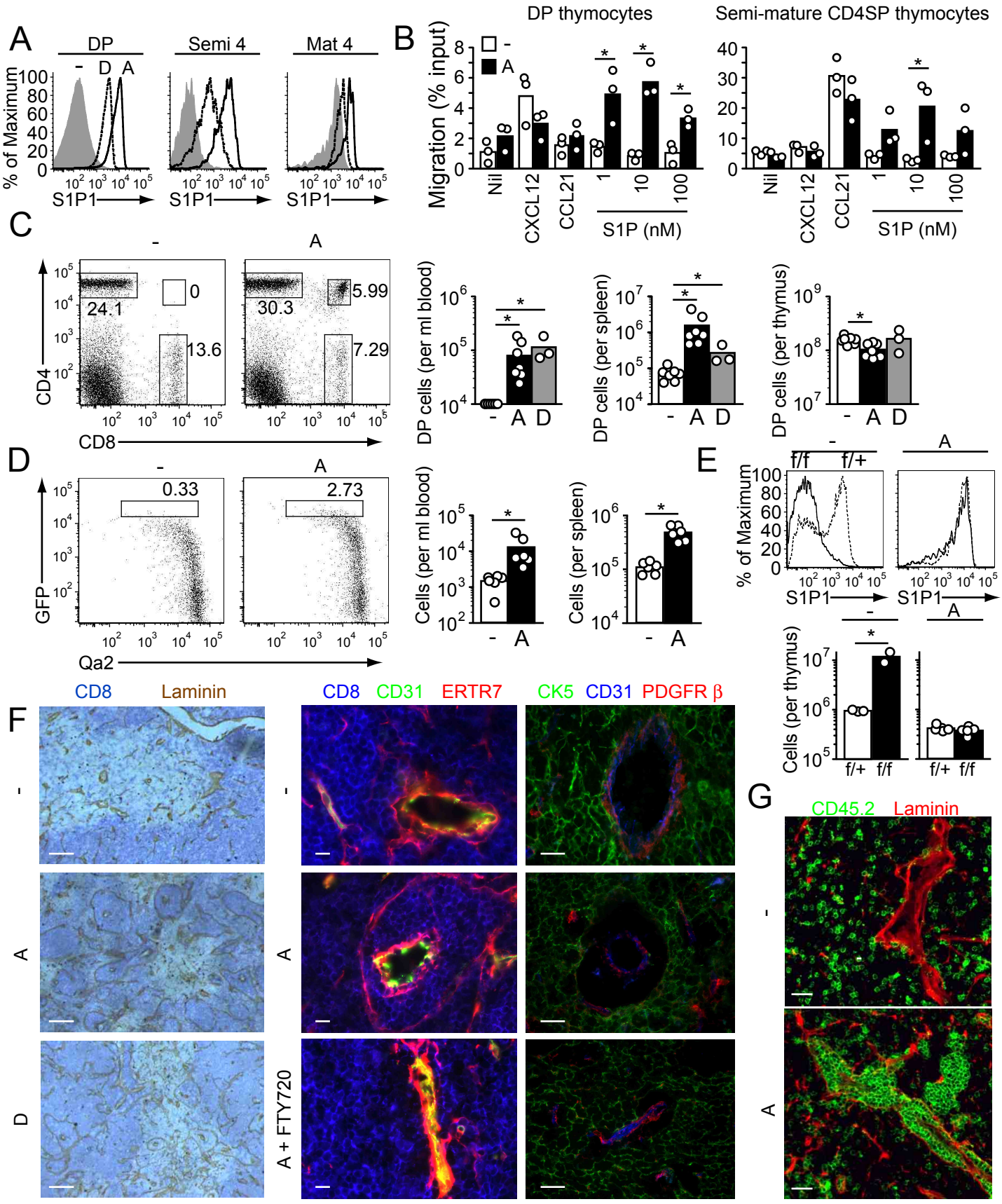
Figure 8. CD69 deficient thymocytes egress prematurely. (a) Flow cytometric analysis of CD69-deficient and control semi-mature and mature thymocytes and enumeration. Semi 4 and Mat 4 correspond to the gates shown in the lower dot plots. Bar graph also shows data for CD94-deficient and control mice. Bars indicate means and circles individual mice. (b) Flow cytometric detection (upper panels) and number (lower panels) of RAG-GFP^{hi} Qa2^{int} recent thymic emigrants in peripheral of CD69-deficient and control mice. (c) GFP intensity of Qa2^{lo}CD62L^{hi} (semi 4), Qa2^{hi}CD62L^{hi} (mat 4) and intravascularly CD4PE labeled thymocytes in wildtype and CD69-deficient RAG-GFP mice. Numbers indicate mean fluorescent intensity. (d) Transwell migration assay of CD69-deficient and control mature CD4 SP thymocytes to S1P, CCL21 and CXCL12. * in A-D indicates $p<0.05$ (student's t-test).

Figure 9. Neural crest-derived pericytes are a necessary source of S1P for thymic egress. (a) Flow cytometric analysis of total thymocytes (left panels) and enumeration of thymocyte subsets (right panels) in the thymus of Wnt1-Cre Sphk-deficient (delta), control (F) and polyI:C treated MxCre Sphk-deficient (MxCre) mice. "n.s." stands for not significant. (b) Flow cytometric analysis (left panel) and enumeration of intravascularly CD4PE labeled cells in Wnt1-Cre Sphk-deficient and control thymi. (c) Reduced number of HSA^{hi}Qa2^{int} recent thymic emigrants in the LNs of Wnt1-Cre Sphk-deficient mice. (d) S1P1 surface abundance on mature CD4 SP thymocytes in the indicated Wnt1-Cre Sphk-deficient or control mice. (e) Reduced frequency of CD69^{lo} mature CD4 SP thymocytes in Wnt1-Cre Sphk-deficient mice. (f) Normal appearance of pericytes in Wnt1-Cre Sphk-deficient mice. Sections were stained to detect the indicated markers. Scale bar indicates 25 μm. (g) Flow cytometric analysis of digested thymus tissue from polyI:C

treated MxCre x RosaYFP reporter mice showing MxCre activity in PDGFRb+CD31- pericytes as well as CD31+GP38- blood vessel endothelium (BEC). CD31+GP38+ lymphatic endothelial cells were undetectable. (h) Flow cytometric analysis of digested tissues from LYVE1-Cre x RosaYFP reporter mice showing CD31 and GP38 staining (left panels) and extent of Cre activity as revealed by YFP expression (right histogram) in blood endothelial cells (BEC) and lymphatic endothelial cells (LEC). (i) Flow cytometric analysis and quantitation showing lack of thymocyte accumulation in LYVE1-Cre Sphk-deficient mice.



Chapter 2, Figure 1



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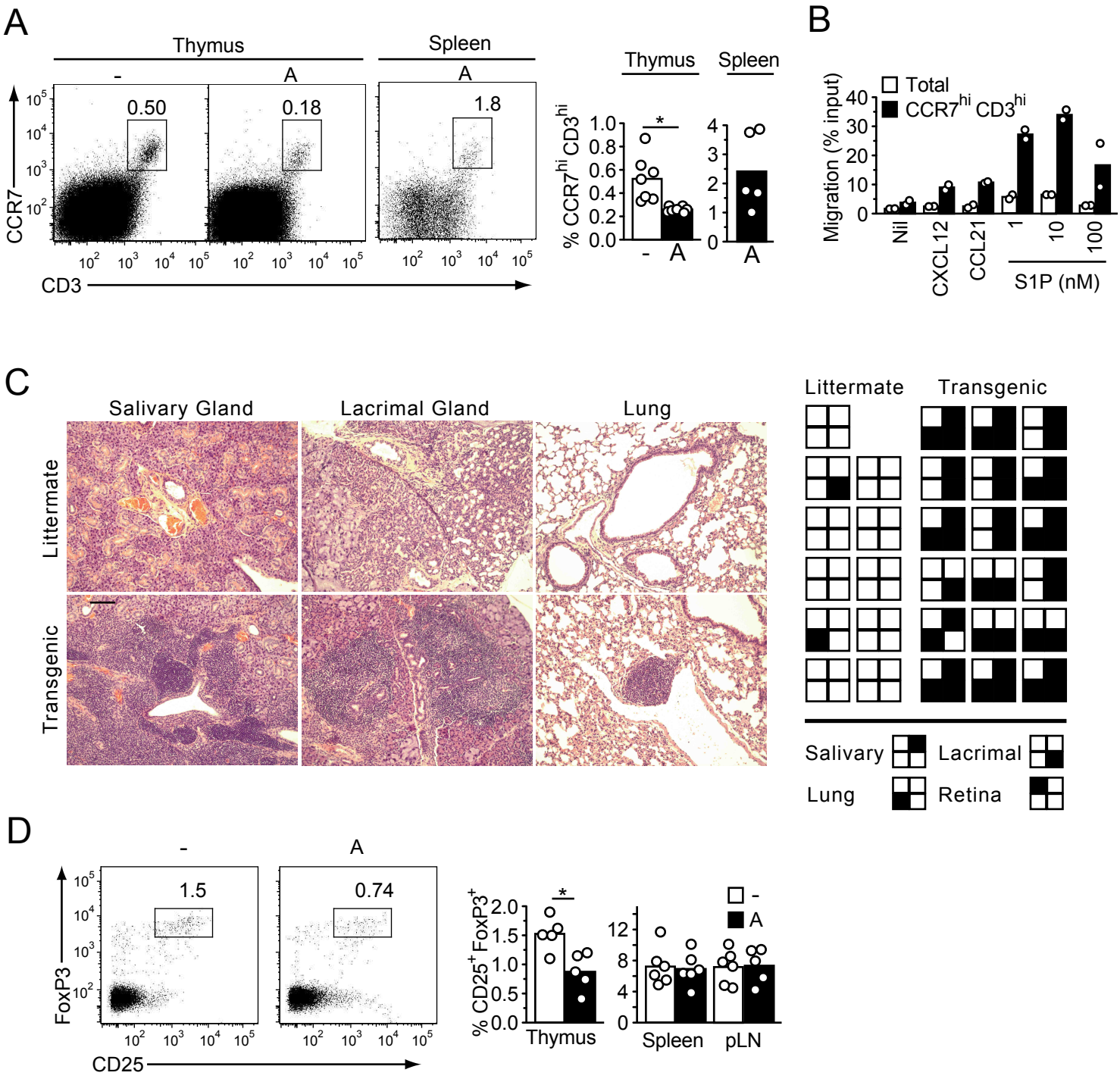
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Thymus
Spleen
-
A
A
0.50
0.18
1.8
CCR7
CD3
Thymus
Spleen
% CCR7hi CD3hi
*
-
A
A
B
Total
CCR7hi CD3hi
Migration (% input)
Nil
CXCL12
CCL21
1
10
100
S1P (nM)
C
Salivary Gland
Lacrimal Gland
Lung
Littermate
Transgenic
Littermate
Transgenic
Salivary
Lacrimal
Lung
Retina
D
-
A
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0.74
FoxP3
CD25
% CD25+ FoxP3+
*
Thymus
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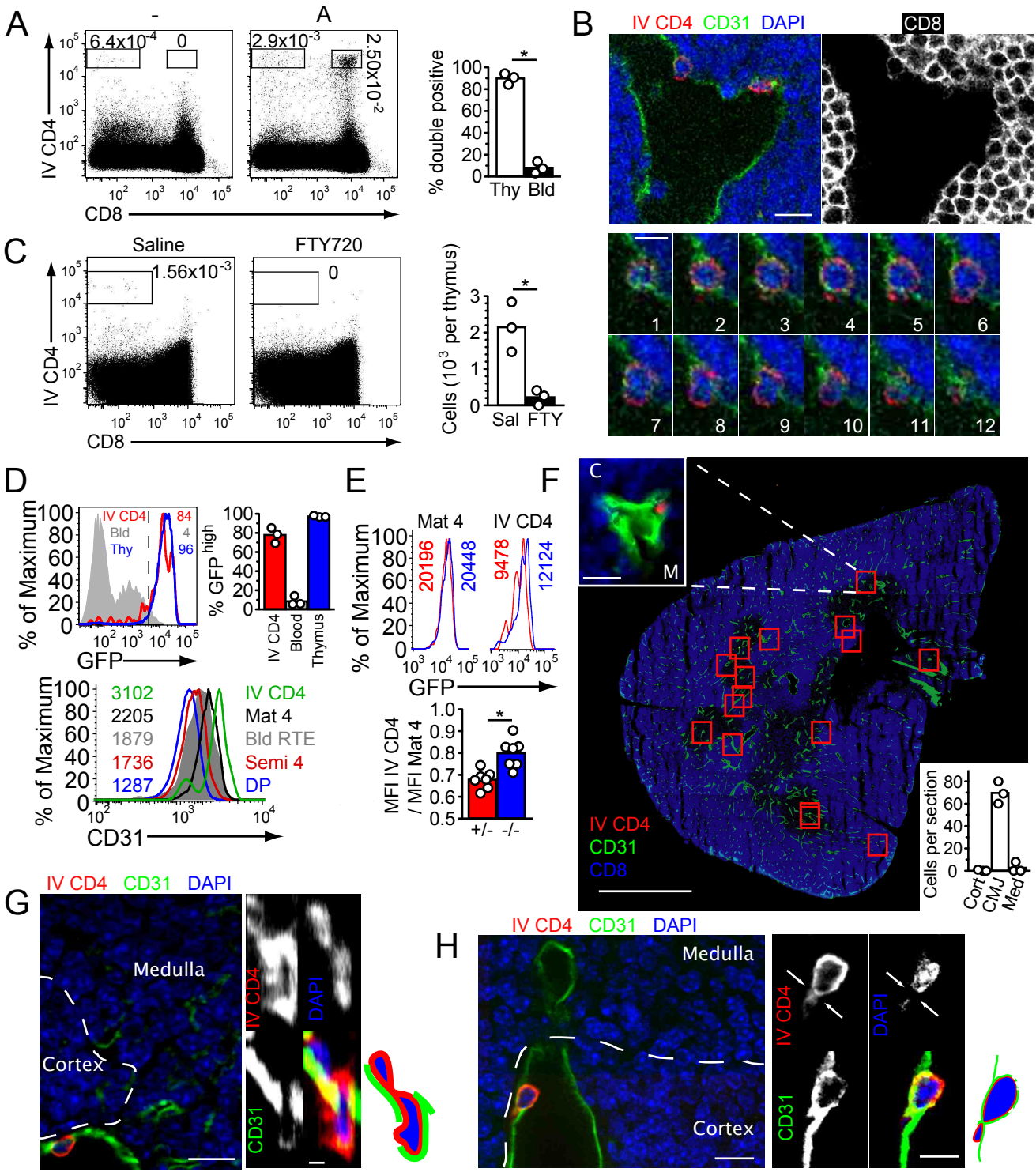
-
A



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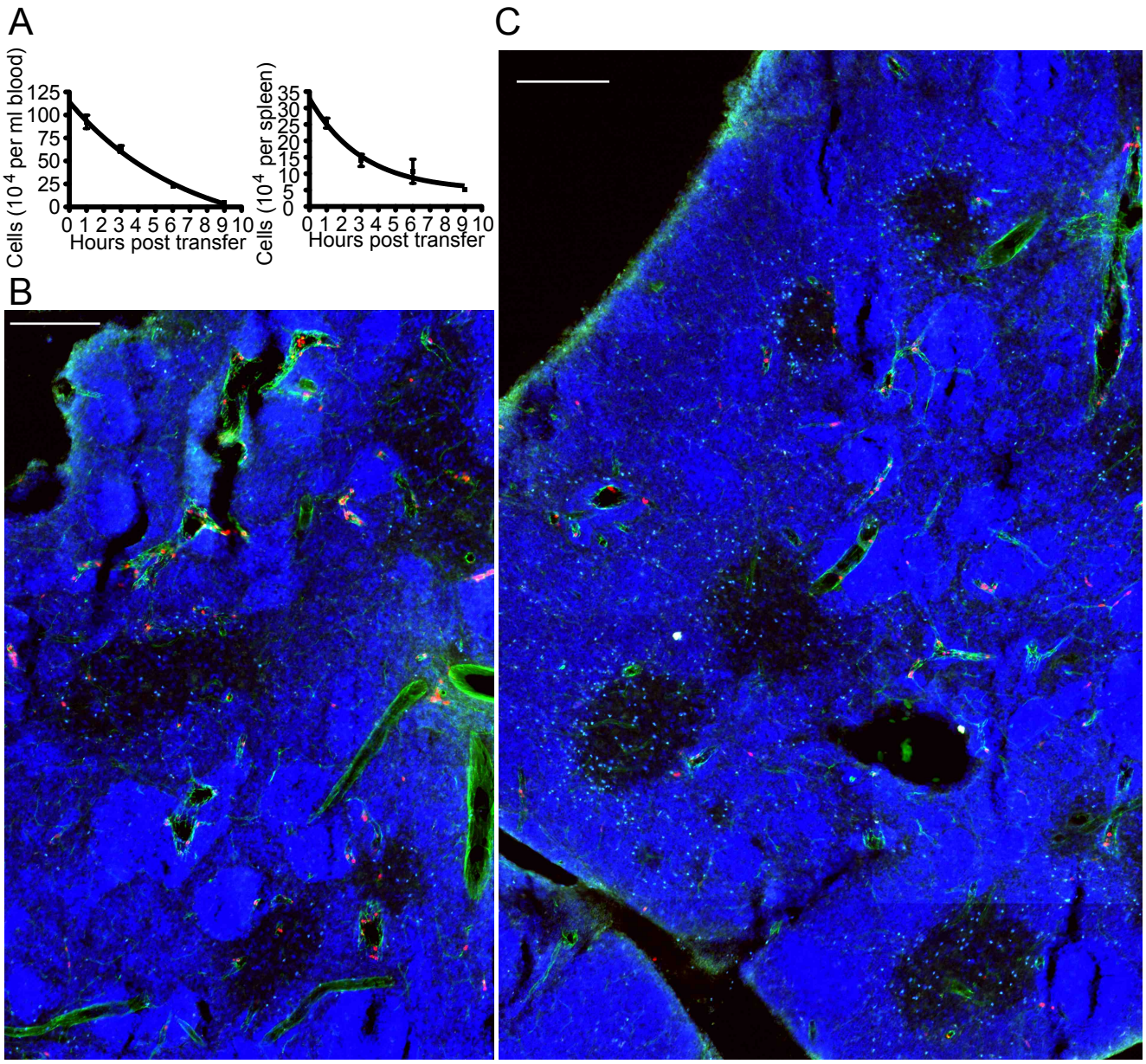

Chapter 2, Figure 5

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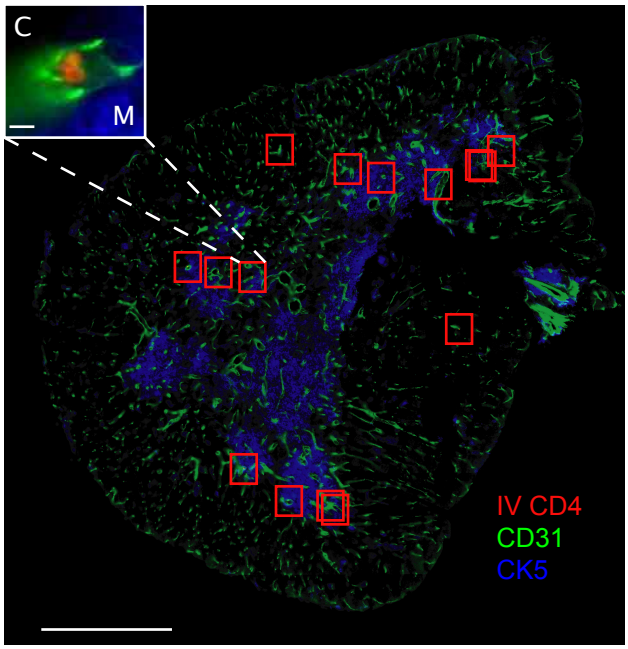

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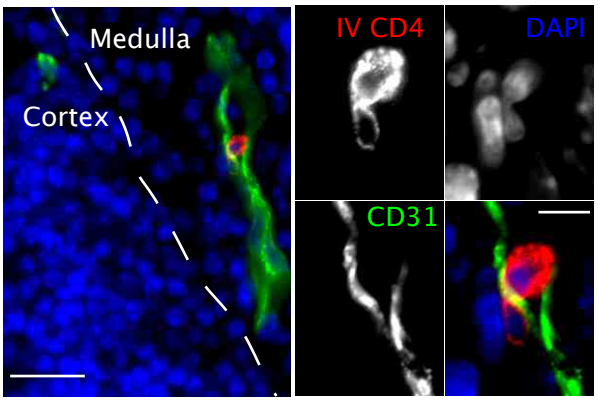

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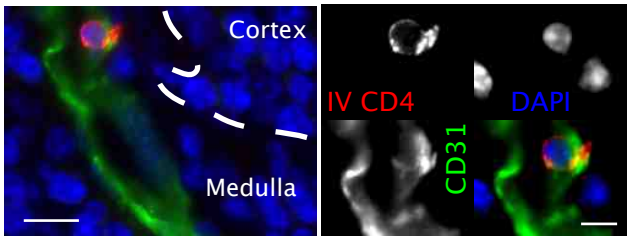
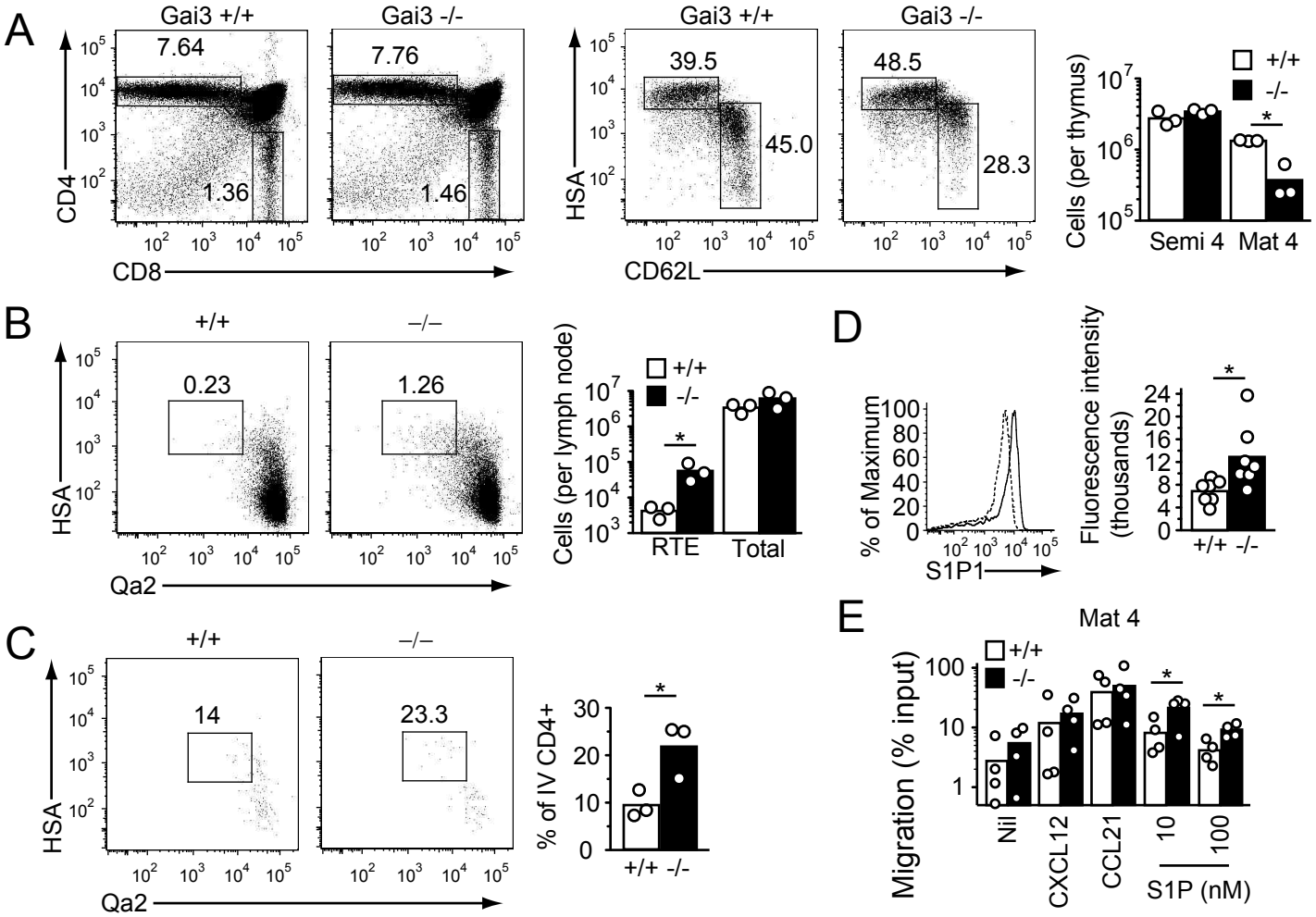


Chapter 2, Figure 6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Gai3 +/+
Gai3 -/-
7.64
1.36
7.76
1.46
CD4
CD8
39.5
45.0
48.5
28.3
HSA
CD62L
Cells (per thymus)
+/+
-/-
Semi 4
Mat 4
B
+/+
–/–
0.23
1.26
HSA
Qa2
Cells (per lymph node)
RTE
Total
C
14
23.3
% of IV CD4+
D
% of Maximum
S1P1
Fluorescence intensity (thousands)
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Mat 4
Migration (% input)
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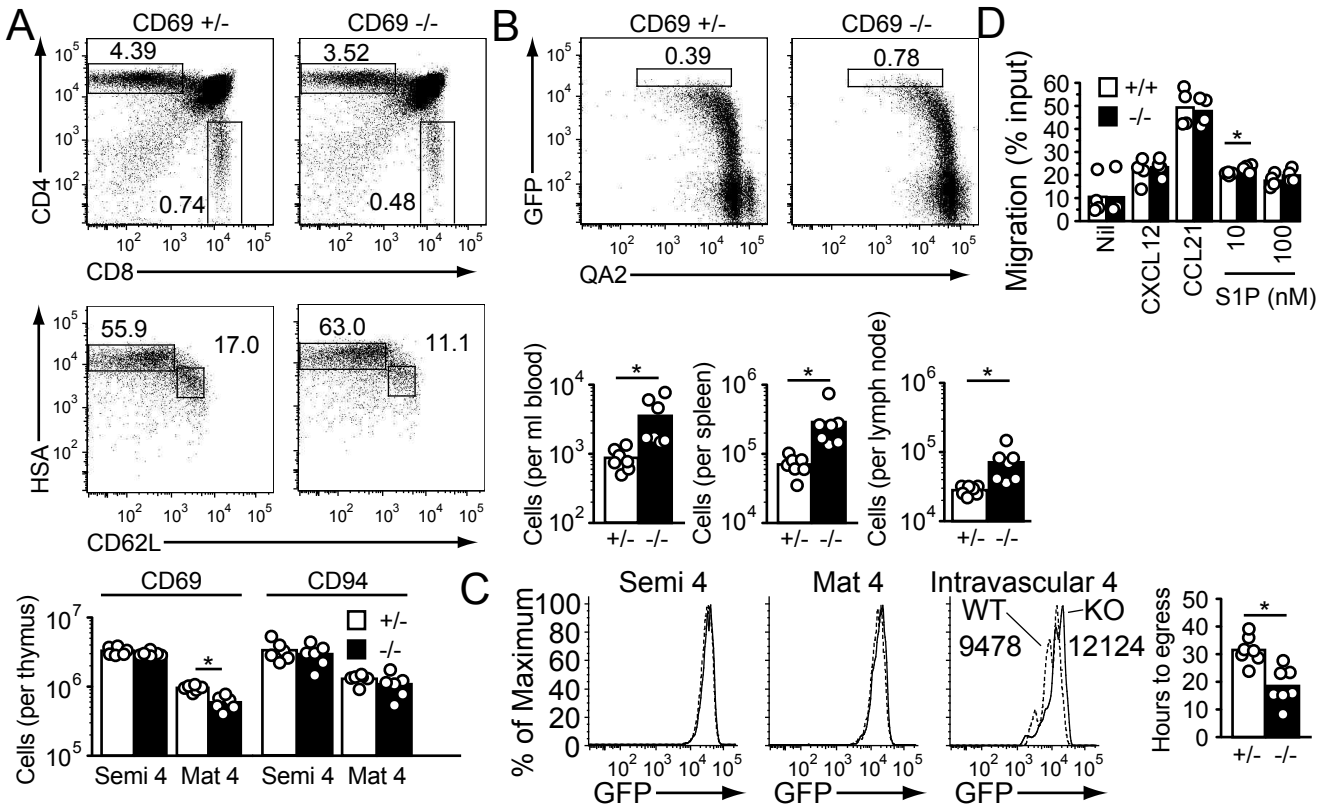
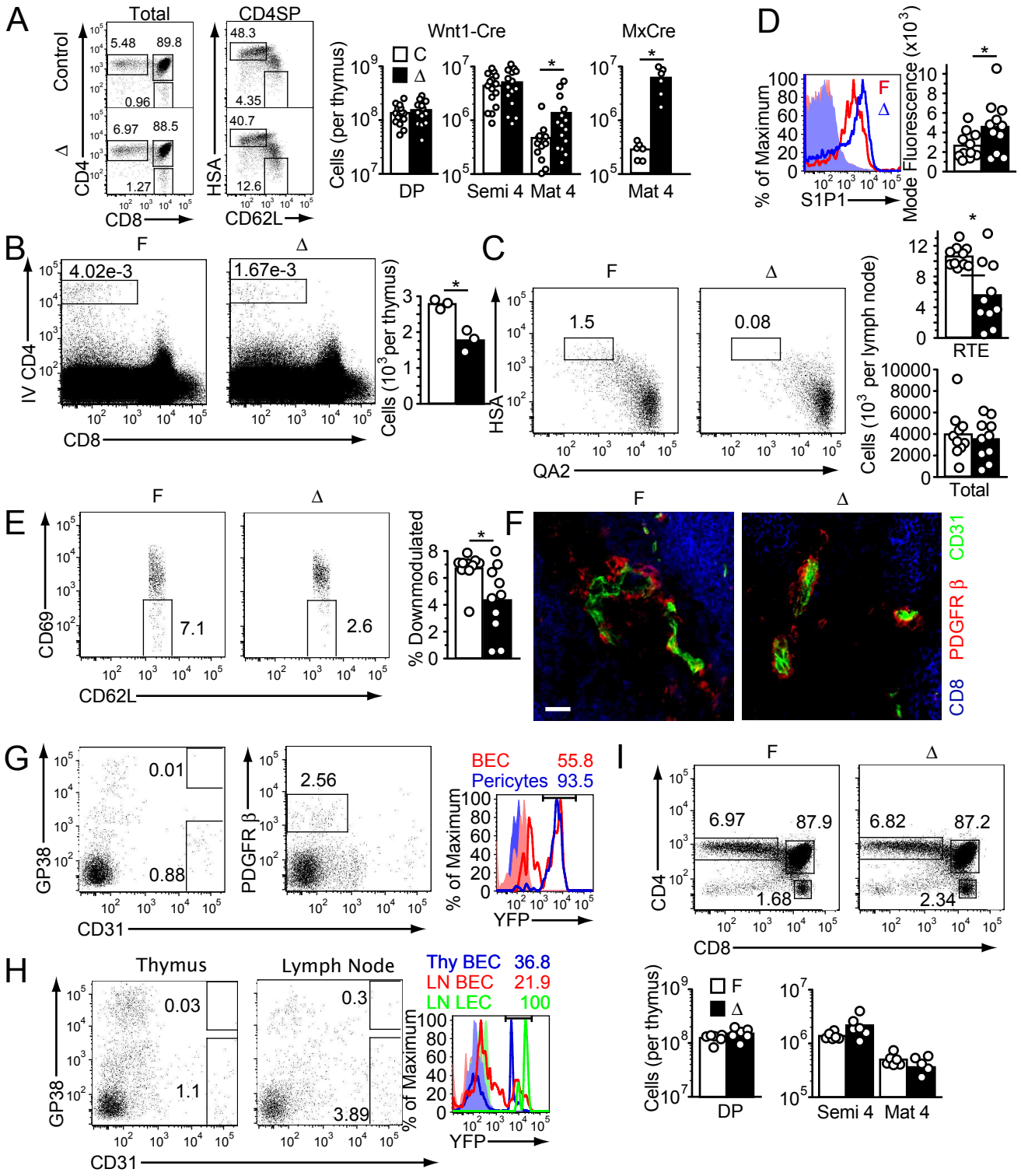


Chapter 2, Figure 8



Chapter 2, Figure 9

CHAPTER 3

Conclusions and Discussion

T cell egress from the thymus is necessary for the generation of an effective adaptive immune system. In the current work, we have provided insight into the mechanism of thymocyte egress and defined the route by which this migration occurs.

Incorporating our data into existing knowledge of thymocyte maturation and egress, we propose the following model. Double-positive thymocytes that undergo appropriate positive selection in the cortex upregulate CCR7 and CD69 and migrate into the medulla as semimature HSA^{hi} $CD62L^{low}$ $Qa2^{low}$ CD4- or CD8-single-positive thymocytes. After 4-5 days time in the medulla, single-positive thymocytes upregulate S1P1 (as well as other genes) under control of the KLF2 transcription factor. S1P1 upregulation is the primary factor controlling medullary dwell time in the thymus, and premature egress may be associated with autoimmune pathology. CD69 expressed on the surface of maturing thymocytes additionally controls timing by inhibiting S1P1-mediated egress, and mature thymocytes only egress with maximum efficiency after fully downmodulating CD69 as $Qa2^{int}$ HSA^{int} $CD62L^{hi}$ $CD69^{low}$ mature single positive thymocytes. Activation of S1P1 by S1P can lead to egress mediated by either Gai2 or Gai3 downstream signaling molecules, but Gai3 additionally acts to negatively regulate egress by promoting S1P1 downmodulation. Egress of medullary mature single-positive thymocytes occurs primarily via reverse transmigration into thin walled, medium diameter corticomedullary blood vessels. Neural crest derived pericytes that enshealth thymic blood vessels contribute to the gradient of S1P that promotes thymocyte egress, whereas lymphatic endothelium does not.

Almost 20 years ago, Chaffin and Perlmutter employed transgenic expression of pertussis toxin to show that thymocyte egress required Gi-mediated signaling (*1*). We

confirmed that thymocyte egress was pertussis-sensitive and demonstrated that the accumulation of mature single-positive thymocytes in LckCre x PTX mice was similar to the accumulation caused by genetic deletion of S1P1 (*2*). This result adds to the body of data suggesting that S1P1 is the major Gi-coupled receptor necessary for egress.

To determine whether S1P1 upregulation is sufficient to account for the acquisition of egress competence by mature single-positive thymocytes, we generated two lines of transgenic mice in which S1P1 is expressed in double-positive and semimature single-positive thymocytes. The migration of double-positive thymocytes into the periphery in these transgenic lines indicated that S1P1 upregulation is sufficient to account for the acquisition of egress competence during thymocyte maturation. Crossing S1P1-transgenic mice to another line in which KLF2 is conditionally deleted in T cells demonstrated that S1P1 is the only KLF2-target that is required for thymocyte egress. Our data do not exclude the possibility of additional genes necessary for egress to occur with maximum efficiency. Indeed the substantial number of double-positive cells remaining in the thymus of transgenic mice and the larger expansion of the perivascular space in the cortex compared to the medulla of transgenic mice hint that egress of cortical thymocytes may be less efficient that egress of medullary thymocytes. This possibility would be consistent with our finding that double-positive transgenic thymocytes chemotax less efficiently to S1P *in vitro* than do single-positive transgenic thymocytes even though the double-positive transgenic thymocytes express higher levels of S1P1. Decreased migration efficiency may be explained by the reduced motility of immature thymocytes, as two photon imaging studies show that cortical thymocytes migrate more slowly that do medullary thymocytes (*3*).

Expression of self-antigens in the thymic medulla by the transcription factor AIRE is important for preventing autoimmune disease in humans and mice (*4*). We hypothesized that S1P1 transgenic mice would exhibit autoimmune disease due to a lack of sufficient exposure to AIRE-dependent and possibly other self antigens presented in the medulla. Indeed, 6-month old S1P1 transgenic mice backcrossed 4 or 5 generations to the C57BL/6J strain suffered from autoimmune infiltrates in salivary gland, lacrimal gland, and lung. However, AIRE-deficient mice do not exhibit infiltrates in lung on a C57BL/6J background (*5*). It will be interesting to examine whether fully backcrossed S1P1 transgenic mice display a different pattern of tissue infiltrates than AIRE-deficient mice. We observed a reduction of thymic T regulatory cell numbers in S1P1 transgenic mice, but the number of the cells in peripheral immune organs was not affected. It will be important to resolve whether autoimmunity in S1P1-transgenic mice results from alterations of regulatory T cell function or from premature egress of effector T cells.

CD69 upregulation on peripheral T cells inhibited egress from lymph nodes (*6*), and CD69 is expressed on semimature and to some extent mature single-positive thymocytes. However, no effect on thymic egress had been seen in CD69 knockout mice (*7, 8*). We hypothesized that CD69 knockout thymocytes might egress prematurely compared to CD69 heterozygous thymocytes. Indeed, knockout mice displayed a loss of mature thymocytes compared to littermate heterozygous controls, and knockout mature single-positive thymocytes migrated more efficiently toward S1P in vitro compared to CD69 heterozygous cells. Crossing CD69 knockout mice with RAG-GFP mice enabled use of GFP intensity as a molecular timer (*9, 10*). CD69 knockout thymocytes exited the thymus several hours earlier than heterozygous thymocytes did, indicating that CD69

contributes to the timing mechanism that holds single-positive thymocytes in the medulla for 4-5days (*10*). Further work to determine whether emigrating CD69 knockout thymocytes are functionally mature and have undergone appropriate negative selection will be necessary to understand the significance of this effect. Additionally, it will be interesting to study the mechanism controlling timing of CD69 upregulation and downregulation in single-positive thymocytes.

The major PTX sensitive Gai-family proteins expressed in T cells are Gai2 and Gai3 (*11-13*). In mice lacking Gai2, thymopoiesis is altered, apparently due to a role for this G-protein in thymocyte maturation rather than an essential role in thymic egress (*14*). Whether Gai3 has a role in thymic egress had not been determined. We demonstrated that genetic deletion of Gai3 leds to a loss of mature single-positive thymocytes and increased cells in peripheral lymph nodes with a less mature HSA^{int} $Qa2^{int}$ profile. Gai3-deficient thymocytes in the act of egress were shown to be less mature in terms of surface phenotype. Increased surface expression of S1P1 on Gai3-deficient thymocytes and increased in vitro chemotaxis toward S1P support a model in which Gai3 negatively regulates egress by downmodulating S1P1. It should be noted that Gai3 may play both egress-promoting and egress-inhibiting roles, because thymocytes deficient in Gai2 (these thymocytes only possess Gai3) apparently egress normally (*14*). Understanding the mechanism by which Gai3 leads to downmodulation of S1P1 is an important future direction.

Studies assessing the route by which T cells emigrate from the thymus began in the 1960s. Electron microscopy work identified cells appearing to cross postcapillary venules at the corticomedullary junction (*15-18*). However, early thymic progenitors are

thought to enter the thymus at this location (*19, 20*), and these studies could not determine whether cells were entering or exiting the thymus. Additionally, other work suggested lymphatics as the major site of thymocyte egress (*18, 21-24*). Whether blood vessels or lymphatics predominated as a site of egress remained unknown.

Single-positive thymocytes are located in the thymic medulla. This would suggest the medulla as the general area where egress occurs. However, work in CCR7-deficient systems, where entry into the medulla is impaired, suggested that egress could occur via the cortex (*25-28*). We sought to identify where egress occurred in the thymus and which vessels were involved.

To do so, we developed a non-invasive technique to enumerate and phenotye cells caught in the act of egress into blood vessels. In S1P1 transgenic mice, egress occurred into blood vessels across the cortex, corticomedullary junction, and medulla. This confirmed earlier work examining CCR7 knockout thymocytes that indicated that egress from the cortex might be possible. The data does not exclude potential differences in efficiency between blood vessels in different locations.

Applying this technique to wildtype C57BL/6J mice, we demonstrated that the vast majority of emigrating thymocytes exited via cortiomedullary blood vessels. Most cells crossed into blood vessels situated no more than 50 microns from the corticomedullary junction. Importantly, the number of cells calculated to egress via corticomedullary blood vessels was close to the number estimated to emigrate from the thymus in total (*29*). This suggested that egress predominantly occurs via corticomedullary blood vessels. Our work indicates that Raviola et al. and other

pioneering electron microscopy studies (*15-18*) may have indeed visualized thymocytes in the act of egress.

Postcapillary venules at the corticomedullary junction are large in diameter, thin walled, and surrounded by a single layer of pericytes. This suggests a possible specialization for egress – future work should examine whether these vessels are specialized in their expression of adhesion molecules or other factors that may be important in egress. Additionally, it will be interesting to examine whether egress occurs via pericellular diapedesis (in between endothelial cell junctions) or via transcellular diapedesis (through the endothelial cells themselves). Both routes are thought to be possible for transmigration in the forward direction, but the route of reverse transmigration is unknown. It is interesting to consider that egress occurs without disrupting the thymus-blood barrier (*15*).

S1P produced by sphingosine kinases in lymphatic endothelium are required for reverse transmigration into lymphatics in the lymph node (*30*). We ablated sphingosine kinases in lymphatic endothelium and saw no effect on egress. This result argues against a major role for lymphatics as a necessary site of egress from the thymus.

Considering egress via corticomedullary blood vessels, we sought to better understand how the gradient of S1P that promotes egress into these vessels is maintained. Previous work showed that S1P is present at high concentration in the blood and low concentration in the thymus (*31*). S1P lyase is required to maintain S1P at a low level in the thymus, and lyase inhibition led to a block in thymic egress. Sphingosine kinases produce S1P, and these kinases are required in MxCre-sensitive cells to keep S1P at a high, egress-promoting level in the blood (*32*). Interestingly, thymocyte egress depended

on the presence of these kinases in radiation-resistant but not radiation-sensitive, MxCre-sensitive cells. When kinases were eliminated from radiation-sensitive, MxCre-sensitive cells, blood S1P levels were normal but thymocyte egress was reduced. This result seemed to conflict with a route of egress from the thymus into the blood.

Pericytes are a diverse population of cells that closely ensheathe blood vessels, but not lymphatics (*33, 34*). Recently it was discovered that thymic pericytes are unusual in having a neural crest origin (*35, 36*). Thymocytes undergoing reverse (basolateral-to-apical) transmigration are likely to encounter pericytes prior to engaging the endothelium. This prediction and our finding that S1P1 transgenic thymocytes accumulate adjacent to pericytes led us to hypothesize that S1P produced by neural crest–derived pericytes might be required for thymic egress.

Wnt1-Cre is an established neural crest specific cre (*37*). To test for a possible role of pericytes as a radiation-resistant S1P source necessary for thymocyte egress, Wnt1-Cre mice were intercrossed with Sphk1f/– Sphk2–/– mice (*32*), to generate mice with neural crest–derived cells lacking the two kinases required for production of S1P.

The accumulation of mature single-positive thymocytes and loss of recent thymic emigrants in Wnt1-Cre Sphk deficient mice suggests that pericytes contribute to the S1P required for egress. Increased S1P1 levels on the surface of mature single-positive thymocytes in Wnt1-Cre Sphk deficient mice support the possibility that sphingosine kinases in pericytes produce S1P that can activate and downmodulate S1P1 present on mature single-positive thymocytes. Reduced CD69 downmodulation on mature thymocytes suggests that pericyte-derived S1P may contribute to downmodulation of CD69 likely in a complex with S1P1 (*6*).

These data help explain the seemingly discrepant findings that thymocyte egress occurs into the blood, yet plasma S1P alone cannot promote normal egress (*32*). Endothelial cells can produce S1P in vitro (*38*) and we do not exclude the possibility that endothelial cell S1P works together with pericyte and plasma S1P in promoting thymocyte egress.

It will be interesting to examine whether pericytes in all tissues secrete S1P or whether this is a unique property of neural-crest derived or thymic pericytes. Additionally, neural crest-derived pericytes are localized nearby the sites of entry into the thymus. Do these cells contribute factors that promote entry of early thymic progenitors? Finally, how complete is the coverage of corticomedullary blood vessels by pericytes. Confocal microscopy (*35, 36*) suggests that the coverage may be quite complete, but electron microscopy will be important in determining whether pericytes form a tight barrier at an ultrastructural level.

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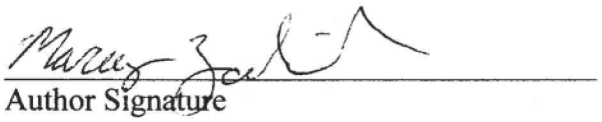

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