Title
Characterization of the mechanism for lymphocyte egress from secondary lymphoid organs

Author
Pham, Trung Hoang Minh

Publication Date
2009

Peer reviewed|Thesis/dissertation

Characterization of the mechanism for lymphocyte egress from secondary lymphoid organs

by

Trung Hoang Minh Pham

DISSTERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

Of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

This dissertation is dedicated to my parents, Duc Pham and Hoa Nguyen, and my seven siblings—Minh Quan, Minh Quoc, Minh Huan, Thuc Chi, Minh Chuong, Minh Tri, and Thao Chi—for their unconditional love and support that allow me to pursue my dreams in science and medicine

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude for the exceptional mentorship and generous support from Dr. Jason Cyster. I was fortunate that he accommodated my oddly timed rotation in his lab while I was a second-year medical student and eventually took me into the fold of his mentorship, where I have learned enormously from his example the skills required for excellence in scientific inquiries. He inspires me with his intellectual and creative capacity, his vast knowledge of scientific literature, and his devotion to the development of the future generation of scientists. I am also grateful for Dr. Cyster's support for the many activities that I did outside of the lab during my training.

I am immensely appreciative of my two other thesis committee members, Drs. Shaun Coughlin and Art Weiss. My graduate training and scientific progress benefited a great deal from their expertise, rigor, and invaluable advice. As a trainee in the Medical Scientist Training Program at UCSF, I am also thankful of Dr. Art Weiss for the efforts he made during his tenure as the director of the program to ensure an excellent training experience for its students. I would also like to thank my qualifying exam committee members, Dr. Shaun Coughlin, Dr. Nigel Killeen, Dr. Steve Rosen, and especially Dr. Tony DeFranco, who generously served as the chairperson of the committee and gave me very thoughtful feedback on my proposals.

I am also indebted to the past and present members of the Cyster lab, who collectively created an exceptional environment that nurtured my development as a scientist. I thank them for intellectual stimulation, invaluable advice, generous support, and friendship. At the beginning of my training in the Cyster lab, I benefited a great deal from the superb administrative support provided by Joan Junkin, who kindness and wonderful cooking I will always remember. I'm thankful of Claire Chan for continuing Joan's work in giving us outstanding support to do our science. Ying Xu is one of the most talented molecular biologist I've met and her baking skills parallel her skills in molecular biology. I am fortunate to have benefited from all those talents of hers. I thank Olivia Lam and Jin-Ping An for giving me excellent help with mouse colonies throughout my studies. I am indebted to Taka Okada, Mehrdad Matloubian, and Charles Lo for helping build the foundation for my thesis studies. Charles was a very generous colleague, from whom I learned a great deal during my rotation in the lab. I was fortunate to have bench and desk space right next to Taka, whose scientific rigor had a strong influence on me. Although I overlapped with Robin Lesley for only a short period of time, I admired the methodical and intelligent way by which she conducted her research. Kenji Kabashima gave me a tremendous amount of help during my early days in the lab, especially with making S1P1-/- fetal liver chimera, an experimental technique that became instrumental to my studies later on. I thank Bryan Cho for thoughtful discussion on scientific and clinical training, Nicole Haynes for her kind and playful personality, and Craig Jenne for being a great ice-hockey reference. Susan

Schwab was a great source of scientific wisdom and activism inspiration. I thank Guy Cinnamon, Tri Phan, and Joao Perreira for sharing their scientific wisdom. I am grateful for Chris Allen's invaluable scientific and technical advices. I was fortunate to follow Larry Shiow footstep as a MSTP trainee in the Cyster lab. I thank him for being a wonderful role model for me and for the fun memories outside of lab. I have a great deal of admiration for Irina Grigorova's love for science, intelligence and charm. I am indebted to her for many useful scientific conversations. In recent time, I have enjoyed tremendously Tal Arnon's sharp mind, candidness, and many stimulating discussions on science and life. I have been fortunate to have Lisa Kelly and Betsy Gray as baymates, two very thoughtful colleagues who are among the most well-read people I've known. I thank Jesse Green for his great music taste and common appreciation for the arts, Alex Bankovich for his vast technological knowledge and for always being ready to help others, and Marcus Zachariah for delicious fruits from his parents' garden and fun parties at his home. I thank Kazuhiro Suzuki for good Japanese food hunts and defying gravity with me at Mission Clips. I thank Xiaoming Wang, the latest addition to our lab, for enthusiastically giving me tips on exploring Shanghai.

I also would like to thank the highly cordial, interactive, and supportive UCSF Immunology community, which I have been fortunate to be a part of. In particular, I thank members of the DeFranco lab, the Killeen lab, the Lanier lab, and the Anderson lab, as well as Piam Vacharotayangul, Julia Gilden and Gianna Hammer, for wonderful interactions over the past few years.

My training would not have been possible with support from the Medical Scientist Training Program and the Tetrad program. I am enormously thankful of the dedication of Jana Toutolmin and Catherine Norton in the MSTP office to the educational and personal well-being of students in the program. I also would like to express my great appreciation of Sue Adams and Danny Dam in the Tetrad office, whose exceptional services to students helped make my graduate training such a wonderful experience.

I cannot imagine my life at UCSF without my classmates: Dan Abelson, Beth Apsel, Brian Berger, Amy Chang, Cathy Collins, Debajyoti Datta, Jay Gardner, Brian Gillis, Ervin Johnson, Ahnika Kline, Sarah Morton, and Davina Wu. They are a group of highly talented and genuinely kind individuals. It has been a privilege to be in their company. I thank them enormously for generous support, wonderful memories, and lasting friendship. I also would like to thank Matt Callahan, Marisol Penazola and Ken Shannahan for being great roommates and friends that make life outside of school so enjoyable.

Finally, I would like to thank my family for their unconditional love that has allowed me to get to where I am today. They are the foundation of all that I am. Because of my parents' courageous decision to leave what they were comfortable with in Vietnam for a promising but uncertain future in America, I have had the opportunity to pursue an education that I could not have imagined even in my dreams. My seven siblings have given me so much in life that I can never sufficiently return. I am immensely thankful of my extended family here in the U.S., particularly my cousins, for their continuing love and support.

CONTRIBUTIONS TO THE PRESENTED WORK

All of the work presented in this dissertation was performed under the direct supervision of Dr. Jason G. Cyster. Additional contributions to specific chapters are described below.

Chapter 2 was published as Pham THM, Okada T, Matloubian M, Charles G. Lo, and Cyster JG. (2008). *S1P1 receptor signaling overrides retention mediated by Gαi-coupled receptors to promote T cell egress*. Immunity. 28(1): 122-133. The initial observations on the role of CCR7 as a retention receptor was made by Dr. Takaharu Okada when he was a post-doctoral fellow in the lab. He collected the data presented in figure 1A & B of this chapter. Dr. Mehrdad Matloubian, another former post-doctoral fellow in the lab, initiated studies to characterize the role of LYVE-1+ cortical sinuses in lymphocyte egress. He prepared the images presented in figure 5A-E, and some of the images he obtained were used for quantitation in figure 5F. I performed all the experiments presented in the rest of the figures. Dr. Charles Lo developed the *in vivo* egress assay with entry blockade and the PTX pulse labeling procedure when he was a graduate student in our lab. I prepared all the figures and Dr. Jason Cyster wrote the initial manuscript for this publication. Dr. Cyster and I revised the manuscript together. Other individuals who contributed to this study are listed under the acknowledgement section for this chapter.

Chapter 3 of this dissertation is being prepared for publication and will be submitted to a journal before I graduate. Dr. Ying Xu and Dr. Jason Cyster

generated the *Lyve-1 Cre* knockin mouse. Dr. Peter Baluk, a colleague of ours at UCSF, performed whole mount immunofluoresence experiments to generate data presented in figure 6 of this chapter. Dr. Ying Xu prepared RNA and performed all the RT-PCR reactions shown in figure 2 from samples I generated. Dr. Irina Grigorova collected the data presented in supplementary figure 3. Dr. Alex Bankovich helped with the sort for lymphatic endothelial cells. Jinping An provided excellent care of the mouse colony. I performed all the experiments presented in the rest of the figures. I prepared all the figures and wrote the manuscript, with revision made by Dr. Jason Cyster and myself. Other individuals who contributed to this study are listed under the acknowledgement section for this chapter.

Characterization of the mechanism for lymphocyte egress from secondary lymphoid organs

By Trung Hoang-Minh Pham

ABSTRACT

Lymphocyte egress from peripheral lymphoid organs during recirculation is essential for normal immune functions, but the mechanisms regulating this process are still incompletely understood. From the lymph nodes, naïve lymphocytes egress into the lymph in a process that requires lymphocyte-intrinsic expression of the sphingosine-1-phosphate receptor (S1P1), a G-protein coupled receptor (GPCR). Furthermore, lymphocyte egress is dependent on a radiation resistant source that maintains the level of S1P1's ligand, sphingosine-1-phosphate (S1P), in the lymph. The cellular source of lymph S1P and how S1P1 acts to promote egress are not defined.

We investigated the hypothesis that lymphocyte S1P1 functions to overcome signals that retain cells in lymphoid tissues. We found that CCR7-deficient T cells exited lymph nodes more rapidly while CCR7 overexpressing cells were retained longer, as compared to wild-type cells. Using the immunosuppressive drug FTY720 to down-modulate S1P1 function, we showed that the requirement of lymphocyte S1P1 for egress was partially relieved by CCR7 deficiency, and more fully relieved by pertussis toxin treatment (PTX) that inactivates lymphocyte Gαi. PTX treatment also restored egress competence in

S1P1-deficient T cells. Furthermore, we found T cell accumulation in the LYVE-1+ cortical sinuses was promoted by S1P1 and antagonized by CCR7 expression intrinsically within the lymphocytes.

Next, we studied the role of lymphatic endothelial cells in generating lymph S1P. We showed that mice with tissue-specific deletion of *Sphk1* by *Cre* expression from the lymphatic vascular endothelium gene-1 (*Lyve-1*) and lacking *Sphk2* had a loss of S1P in the lymph while maintaining normal plasma S1P level. In lymphatic *Sphk*-deficient mice, egress from lymph nodes and Peyer's patches was markedly reduced, while nodal LYVE-1+ cortical sinuses lacked lymphocytes and appeared collapsed. Treatment with PTX to inhibit lymphocyte-Gαi restored lymphocyte egress in these mice. Furthermore, we found in the absence of lymphatic *Sphks,* lymphatic vasculature architecture was altered.

Together our findings support a model whereby lymphatic endothelial cells produce lymph S1P, which acts directly on lymphocyte S1P1 to overcome retention signals to promote lymphocyte egress from lymph nodes and Peyer's patches.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

Introduction

Adaptive immune responses are dependent upon the recognition of a particular foreign antigen by a few specific T and B lymphocytes within a large repertoire. Secondary lymphoid organs (SLOs) facilitate this recognition by serving as central stations where T and B cells can survey a collection of captured antigens (Cyster, 2005). For example, lymph nodes filter lymph drainage and present antigens from skin and mucosal surfaces while the spleen is dedicated to displaying blood borne antigens (Cyster, 2005). Thus, the recirculation of lymphocytes among SLOs to survey for antigens is crucial for normal immune responses. A series of insightful experiments by Gowans and colleagues in the 1960s showed that in recirculation, lymphocytes travel from blood to lymph nodes and then back into circulation via the lymph (Gowans, 1996; Gowans and Knight, 1964). Lymphocytes enter the lymph nodes and Peyer's patches through the high endothelial venules (HEV) (Cyster, 2005; von Andrian and Mempel, 2003). The exact routes by which lymphocytes exit the lymph nodes are still not well-defined, although it has been thought that cells leave via the medullary lymphatic sinuses, where they can be carried away by the efferent lymph (Cyster, 2005; Picker, 1999; von Andrian and Mempel, 2003). In the Peyer's patches, cells have been observed to migrate into a network of lymphatic vessels surrounding lymphoid structures (Azzali, 2003). Recent imaging studies show that T cells enter the splenic white pulp (WP) from the marginal sinus where they can gain access into the periarteriolar lymphoid sheath via the marginal zone bridging channels and subsequently migrate along the fibroblastic recticular cell (FRC) network into the deep T zone (Bajenoff et al.,

2008). The exit pathway from the spleen is still poorly defined, although T cells have been observed to leave the WP via the bridging channels to reach the red pulp, where they may enter circulation through venous sinuses (Khanna and Lefrancois, 2008; Mueller and Ahmed, 2008).

Lymphocytes enter the lymph nodes through a series of concerted molecular events that include recognition of endothelial addressins by lymphocyte selectins, rolling along the endothelium, activation by chemokines, firm adhesion, and transmigration (von Andrian and Mempel, 2003). Upon entry, lymphocytes transverse the inter-follicular paracortex before T and B cells move deeper into their respective T and B zones. Lymphoid tissues are rich in chemokines, many of which function as chemoattractants that promote lymphocyte migration and distribution in distinct territories (Cyster, 2005). CCR7, for example, has been shown to promote T cell motility and distribution within the T zone, aside from its role in promoting lymphocyte entry into lymphoid tissues (Asperti-Boursin et al., 2007; Forster et al., 1999; Okada and Cyster, 2007; Worbs et al., 2007). Naïve T and B cells spend about 6-12 or 24 hours, respectively, in the lymph nodes after entering from blood (Cyster, 2005). During this time they migrate continuously within their respective microdomains in a manner that appears “random” but may be guided by paths through networks of stromal cells—fibroblastic reticular cells (FRCs) in the T zone and follicular dendritic cells in the B zone—and local chemoattractant gradients (Bajenoff et al., 2006; Beltman et al., 2007; Cahalan and Parker, 2008; Huang et al., 2004; Miller et al., 2002). Naïve lymphocytes entering the lymph nodes that fail to

detect antigens will return to the circulation via the lymph. An encounter with antigen will result in selective retention of the antigen-specific lymphocytes in the lymph node where they undergo proliferation and differentiation into effector cells (Arnold et al., 2004; Cyster, 2005).

While the molecular mechanisms involved in lymphocyte entry into lymphoid tissue have been well characterized, the steps of lymphocyte egress and their regulation have only recently begun to be illuminated. The discovery that the immunosuppressant drug FTY720 inhibits egress has led to the identification of the first molecular requirement for lymphocyte egress from peripheral lymphoid organs and the beginning of our understanding of the mechanisms that govern this cellular process (Mandala et al., 2002). A synthetic derivative of the fungal metabolite myriocin, which was identified as a potent inhibitor of mixed lymphocyte reaction (Fujita et al., 1994), FTY720 was selected in a screen for its immunosuppressive properties in preventing skin allograft rejection (Fujita et al., 1996). However, it appeared that the mechanism of action for FTY720 *in vivo* was not due to interfering with lymphocyte immuno-reaction, as the dose at which the drug prevented skin-graft did not block mixed lymphocyte reaction, but rather due to its ability to alter the lymphocyte trafficking patterns (Chiba, 2005; Schwab and Cyster, 2007). When administered *in vivo*, FTY720 rapidly depleted naïve lymphocytes from the blood and lymph, with cell numbers in these two compartments reduced by 10-100 fold and a concomitant slight increase in lymphocyte numbers in the lymph nodes and Peyer's patches (Chiba et al., 1998). Since naïve lymphocytes enter the lymph by exiting

lymphoid organs, these findings are consistent with the idea that FTY720 blocks lymphocyte egress from lymphoid organs. Subsequent studies demonstrated that CD62L-low T cells and CCR7-deficient lymphocytes were less efficiently depleted from the blood by the drug, suggesting that FTY720 exerts its effect by redistributing lymphocytes from the circulation into lymphoid organs and sequestering them there (Bohler et al., 2004; Cyster, 2005; Henning et al., 2001). Furthermore, FTY720 was found to inhibit the release of antigen activated T cells from draining lymph nodes while having no detectable effect on T cell proliferation *in vivo* (Rosen et al., 2003).

A significant advance in our understanding of the molecular mechanism of FTY720's action was made with the realization that phosphorylated FTY720 was structurally similar to S1P, an endogenous bioactive sphingolipid (Mandala et al., 2002). FTY720 was found to be a pro-drug, which following conversion into the active phospho-form, acts as an agonist on four of five G-protein receptors that bind to S1P: S1P1, S1P3, S1P4, and S1P5 (Mandala et al., 2002). Later, the phosphorylation of the drug *in vivo* was shown to require SPHK-2, one of two sphingosine kinases that generate cellular S1P from sphingosine (Allende et al., 2004b; Kharel et al., 2005; Zemann et al., 2006). In support of the view that FTY720 inhibits egress through its action on S1P-receptors, intravenous administration of S1P also resulted in depletion of lymphocytes in the blood and thoracic duct (Mandala et al., 2002). Genetic studies have demonstrated an essential role of the S1P1 signaling pathway in lymphocyte egress (Allende et al., 2004a; Matloubian et al., 2004). Ubiquitous gene-targeted deletion of *S1p1*

resulted in embryonic lethality at around E12.5-E13.5 due to vascular defects (Liu et al., 2000). Using fetal liver chimera generated from S1P1-deficient embryos and mice in which *S1p1* is conditionally deleted in T cells, it has been shown that S1P1 is required intrinsically within the lymphocytes for egress from the thymus and peripheral lymphoid organs (Allende et al., 2004a; Matloubian et al., 2004). Mature single-positive thymocytes egress from the thymus to populate the peripheral lymphocyte compartment and then recirculate through SLOs to carry out immunosurveillance. Similar to its dependence on S1P1, egress from the thymus is also blocked by FTY720 (Yagi et al., 2000). Further evidence demonstrating the central role of S1P1 signaling in lymphocyte egress has come from studies involving other pharmacological agents. S1P1-specific agonists, such as SEW2871, have been shown to impair lymphocyte egress (Sanna et al., 2006; Wei et al., 2005). It has been postulated that FTY720 blocks lymphocyte egress by acting as a functional antagonist, as exposure to the drug can result in rapid down-modulation of lymphocyte S1P1 and render cells in a "pharmacological null" state (Cyster, 2005; Matloubian et al., 2004; Schwab and Cyster, 2007).

Consistent with the lymphocyte-intrinsic requirement of S1P1 for egress, lymphocyte S1P1 expression appears to be a major point of egress regulation in various physiological settings. KLF2, a transcription factor expressed in egress-competent mature but not immature thymocytes, is required for efficient expression of S1P1 mRNA. Thymocytes deficient in KLF2 exhibit a thymic egress defect (Carlson et al., 2006). Early in an immune response, lymphocyte

egress from draining lymph nodes undergoes a transient block triggered by various inflammatory stimuli including interferon-α/β, an event that has been thought to increase the frequency of antigen-specific cells inside the nodes. Recent studies provide evidence indicating that this egress block is due to CD69-mediated down-modulation of surface S1P1 (Shiow et al., 2006). At later stages of the immune response, antigen-specific lymphocytes are selectively retained in the lymph nodes where they undergo proliferation and differentiation into effector cells. This selective sequestration can be explained by activation-induced changes in lymphocyte S1P1 mRNA expression, which is down-modulated 50-100 fold one day after T cells are stimulated via their T cell receptor (Matloubian et al., 2004). Concomitantly, at this time point the ability of activated T cells to migrate to S1P is reduced (Matloubian et al., 2004). At day 3 after activation, antigen specific T cells start to recover their S1P1 mRNA expression, as well as their responsiveness to S1P, and begin to reappear in circulation (Arnold et al., 2004; Matloubian et al., 2004). Despite all the findings on the requirement of S1P1 for egress, how lymphocyte S1P1 acts to promote egress remains unclear.

Studies of the physiological role of S1P *in vivo* have provided further understanding on how lymphocyte egress is regulated. Cellular S1P is a product of sphingolipid metabolism and its generation from the immediate precursor sphingosine requires sphingosine kinases (SPHK)-1 and -2 (Kihara and Igarashi, 2008; Kono et al., 2008). S1P is dephosphorylated to regenerate sphingosine by the S1P-dedicated intracellular enzymes S1P-phosphatase (SPP)-1 and -2, or by general lipid phosphate phosphatase (LPP) ectoenzymes (Kihara and Igarashi,

2008). S1P can also be irreversibly degraded into hexadecanal and phosphoethanolamine through the action of S1P lyase (Kihara and Igarashi, 2008). Although S1P is thought to have roles as an intracellular signaling molecule, the lack of identified intracellular targets of S1P has hampered our understanding of how S1P functions in this capacity (Spiegel and Milstien, 2003). In contrast, S1P's function as an extracellular signaling molecule through its receptors has been better characterized (Kono et al., 2008). *In vivo*, the S1P level is normally low in lymphoid tissue and high in the blood and lymph, and the gradient of S1P between tissues and circulatory fluids has been shown to be important for lymphocyte egress (Pappu et al., 2007; Schwab et al., 2005). In a series of elegant experiments, it was found that inhibition of S1P lyase activity *in vivo* leads to elevated lymphoid tissue S1P, disruption of the circulation/tissue S1P gradient, and lymphocyte egress block (Schwab et al., 2005). Similarly, egress is inhibited in mice that have low level of S1P both in the tissue and in circulation as a result of gene targeted deletion of *Sphk-1* and *-2* (Pappu et al., 2007).

As the role of S1P/S1P1 signaling in lymphocyte egress is being uncovered, a debate has emerged on whether this signaling pathway acts on the lymphocytes or on stromal cells to regulate lymphocyte egress (Cahalan and Parker, 2008; Rosen et al., 2007; Schwab and Cyster, 2007). In one model for how S1P/S1P1 signaling regulates egress, it is postulated that stimulation of S1P receptor(s) on endothelial cells forming the egress barrier between lymphoid tissues and the blood or lymph causes tightening of the endothelium and

prevents egress. Data used to support this model come mainly from studies involving the use of S1P agonists to perturb lymphocyte trafficking, in which the drugs were shown to cause a “log jam” of lymphocytes at the medullary sinuses and clearance of these vessels of cells (Mandala et al., 2002; Sanna et al., 2006; Wei et al., 2005). However, in all these studies the effects of agonists of S1P receptors were not specific to endothelial cells, leaving the possibility that actions of the drugs on other cells, such as lymphocytes could account for the egress inhibitory effect of the drugs. In an alternative model, S1P/S1P1 signaling is hypothesized to act intrinsically within the lymphocytes to promote egress from lymphoid organs by overcoming a retention signals that normally retains cells in the tissues or by mediating directional migration of cells into the lymph (Schwab and Cyster, 2007). Consistent with this model, S1P1-/- T and B cells transplanted into wild-type recipients showed an egress defect (Matloubian et al., 2004). In lymphocytes that lost S1P1 expression and egress competence, restoration of lymphocyte S1P1 function led to recovery of egress competence (Lo et al., 2005; Shiow et al., 2006). Furthermore, the findings that disruption of the S1P gradient between circulatory fluids and lymphoid tissues blocked lymphocyte egress, both when the S1P level is equally low and equally high across these two compartments, are more consistent with a mechanism where directional cell movement is promoted by S1P/S1P1 signaling, rather than an endothelial cell gating mechanism (Pappu et al., 2007; Schwab et al., 2005). Although the two models described above are in principle not mutually exclusive, understanding how S1P signaling acts within the lymphocytes to promote egress

may help determine which model better captures the mode of lymphocyte egress regulation by S1P/S1P1 signaling across various settings and help resolve this debate.

S1P and the five receptors, S1P1-5, on which it acts are involved in a wide-range of biological processes (Kono et al., 2008), yet not much is known about the *in vivo* sources of extracellular S1P that mediate different physiological processes. The cellular sources of S1P relevant for lymphocyte egress have begun to be identified. Recently, it was found that about 95% of plasma S1P is supplied by hematopoietic cells, with red blood cells (RBCs) being a major contributor; however, lymph S1P appears to be maintained by a distinct, radiation resistant cell type. Evidence for this comes from experiments in mice lacking circulatory fluid S1P and having a block of egress from lymphoid organs. Transplantation of wild-type bone marrow or wild-type (RBCs) into these mice restored plasma S1P but not lymph S1P, which correlated with restoration of egress from the thymus and spleen but not from lymph nodes (Pappu et al., 2007). These findings suggest that hematopoietic cells, particularly RBCs, are a cellular source of S1P relevant for egress from the thymus and spleen into the blood. However, the source of lymph S1P required for lymphocyte egress from the lymph nodes remains to be defined, though it has been speculated that lymphatic endothelial cells may be such a source (Schwab and Cyster, 2007).

Lymphatic endothelial cells (LECs) are thought to arise from the venous endothelium during embryonic development around E9.0-9.5, when a subpopulation of endothelial cells of the anterior cardinal vein commit to the

lymphatic lineage by turning on Lyve-1 and *Prox-1* expression (Karpanen and Alitalo, 2008; Oliver and Srinivasan, 2008). LYVE-1 is the earliest expressing and one of the most specific and widely used markers for LECs. A homeobox master regulator of lymphatic endothelial differentiation, PROX-1 subsequently promotes expression of a number of LEC selective genes while suppressing expression of genes characteristic of blood endothelial cell (BEC) cell fate. Comparative analysis of global gene expression between LECs and BECs shows proteins involved in transport and secretion are highly represented in LECs, suggesting these cells might be an important source of extracellular molecules (Podgrabinska et al., 2002).

S1P signaling has a central role in the development of the cardiovascular system. Mice lacking *Sphk1* and *Sphk2* die *in utero* between E11.5-E13.5 due to vascular defects that result in widespread hemorrhages (Mizugishi et al., 2005). Similarly, gene-targeted deletion of S1P1 results in embryonic lethality around E12.5-E13.5, also due to vascular defects (Liu et al., 2000). *In vitro*, stimulation of endothelial cells with S1P increases localization of VE-Cadherin at cell-cell junctions and induces tubular morphogenesis (Lee et al., 1999). Furthermore, S1P potentiates VEGF-induced angiogenesis *in vivo* in a matrigel plug assay (Lee et al., 1999). Whether S1P signaling plays a role in the development of the lymphatic system is not known. Recently, S1P was demonstrated to promote tubular formation of human lymphatic endothelial cells *in vitro* and lymphangiogenesis in matrigel *in vivo* (Yoon et al., 2008). In addition, S1P stimulates angiopoietin-2 exocytosis in lymphatic endothelial cells in an S1P1-

dependent manner (Jang et al., 2009). Angiopoietin-2 has been shown to play an important role in lymphatic development as gene-targeted deletion of *Angpt2* in mice leads to defects in lymphatic architecture and lymphedema (Gale et al., 2002).

Despite the evident importance of S1P signaling in development and for normal lymphocyte egress in the adult organism, our understanding of the *in vivo* mechanism of control of lymphocyte egress from secondary lymphoid organs remains incomplete. The studies presented in this dissertation use a variety of approaches to illuminate the cellular and molecular mechanisms that regulate this process.

CHAPTER 2

S1P1 receptor signaling overrides retention mediated by Gαi-coupled receptors to promote T cell egress

This chapter was published as:

Pham THM, Okada T, Matloubian M, Lo CG, Cyster JG. (2008). S1P1 receptor signaling overrides retention mediated by Gαi-coupled receptors to promote T cell egress. *Immunity*. **28**(1): 122-33. Epub 2007 Dec 27.

Summary

The mechanism by which sphingosine-1-phosphate receptor-1 ($S1P_1$) acts to promote lymphocyte egress from lymphoid organs is not defined. Here we showed that CCR7-deficient T cells left lymph nodes more rapidly than wild-type cells whereas CCR7 overexpressing cells were retained for longer. After treatment with FTY720, an agonist that causes down-modulation of lymphocyte $S1P_1$, CCR7-deficient T cells were less effectively retained than wild-type T cells. Moreover, treatment with pertussis toxin to inactivate signaling via Gαi-protein coupled receptors restored egress competence to $S1P_1$ deficient lymphocytes. We also found that T cell accumulation in lymph node cortical sinusoids required intrinsic $S1P_1$ expression and was antagonized by CCR7. These findings suggest a model where $S1P_1$ acts in the lymphocyte to promote lymph node egress by overcoming retention signals mediated by CCR7 and additional Gαi-coupled receptors. Furthermore, by simultaneously upregulating $S1P_1$ and downregulating CCR7, T cells that have divided multiple times switch to a state favoring egress over retention.

Introduction

Lymphocyte egress from lymphoid organs is essential for immunsurveillance and for lymphocyte effector function. After entry into lymphoid tissues, lymphocytes spend several hours to a day migrating extensively within the tissue prior to exiting and returning to circulation (Cyster, 2005). Lymphocyte entry into lymph nodes occurs via high endothelial venules (HEVs) and requires the concerted action of selectins, chemokines and integrins (von Andrian and Mempel, 2003). The principal chemokine receptor involved in entry, CCR7, is highly expressed on T cells and the ligand, CCL21, is abundant on HEV and is present on stromal cells throughout the T zone. The second CCR7 ligand, CCL19, is also expressed by T zone stromal cells (Cyster, 2005). Recent work has shown that CCR7 and CCR7 ligands are not only involved in cell entry to lymphoid tissues but they also promote T cell motility within the T zone (Huang et al., 2007; Okada and Cyster, 2007; Worbs et al., 2007).

In contrast to the established cascade of molecular interactions required for cell entry from blood into tissues, the multistep requirements for egress have not been well defined. Egress occurs from lymph nodes and Peyer's patches into efferent lymphatics and from spleen into blood (Cyster, 2005). The exact site of egress in each of these tissues is not firmly established although egress from lymph nodes is typically thought to occur at the medullary sinuses that connect to the subcapsular space and the efferent lymphatic (Sanna et al., 2006; Wei et al., 2005). An additional sinus structure that begins in the T zone and joins with the medullary sinuses, termed the cortical sinusoid, was also suggested based on

ultrastructural studies to be involved in egress (Kelly, 1975; Soderstrom and Stenstrom, 1969). However, the role of cortical sinusoids in egress has not been further explored.

Important insight into molecular requirements for lymphocyte egress came from the finding that the immunosuppressant molecule, FTY720, inhibited egress (reviewed in (Cyster, 2005; Rosen et al., 2007)). After in vivo treatment, FTY720 becomes phosphorylated and the phospho-form is an agonist on four of the five sphingosine-1-phosphate (S1P) receptors including S1P receptor-1 ($S1P_1$), a receptor that signals via Gαi-containing heterotrimeric G-proteins (Rosen et al., 2007). Genetic studies have established that $S1P_1$ is needed in the lymphocyte for normal egress to occur from thymus and peripheral lymphoid tissues (Allende et al., 2004; Matloubian et al., 2004). Recent work has shown that S1P is required for induction of egress (Pappu et al., 2007) and treatments that cause elevations in lymphoid tissue S1P abundance disrupt egress (Schwab et al., 2005). Despite these advances, the mechanism by which $S1P_1$ acts in the lymphocyte to promote egress has been unclear.

In earlier work, we suggested that FTY720 acts to inhibit egress by down-regulating and functionally antagonizing lymphocyte $S1P_1$ (Matloubian et al., 2004). However, engagement of endothelial $S1P_1$ can increase tight junction formation and it has been proposed that FTY720 may instead inhibit egress by closing egress portals (Sanna et al., 2006; Wei et al., 2005). Thus, the mechanism by which FTY720 and other $S1P_1$ agonists inhibit lymphocyte egress is still debated (Rosen et al., 2007).

Two non-mutually exclusive models can be considered for how lymphocyte $S1P_1$ promotes egress: (1) it functions to overcome signals that retain cells in the lymphoid tissue; (2) it transmits unique signals that promote migration or transmigration into exit structures. Here we have performed experiments that test the first of these models. We demonstrate that CCR7 and additional Gαi-coupled systems promoted T cell retention within lymphoid organs and that lymphocyte $S1P_1$ functioned to overcome these retention signals. Inhibition of lymphocyte Gαi overcame FTY720 mediated retention, providing further evidence that this drug inhibits egress by acting on the lymphocyte. Lymph node cortical sinusoids were identified as a site where $S1P_1$ acted during lymphocyte egress from lymph nodes. Together these studies provide evidence for a model where $S1P_1$ functions to promote egress at least in part by overcoming signals that retain cells in the lymph node.

Results

CCR7 promotes T cell retention in lymph nodes

To test whether CCR7 can mediate T cell retention in lymph nodes, CCR7 deficient and wild-type splenocytes were co-transferred into wild-type recipient mice and equilibrated for one day. Circulating lymphocytes were then inhibited from further entry into lymph nodes by treating the mice with integrin-neutralizing antibodies (‘entry blockade’), and co-transferred cells were enumerated either at the treatment time (t = 0 h) or eight hours later (t = 8 h). Previous studies have shown that aL- and a4-containing integrins are not required for lymphocyte

egress (Arnold et al., 2004; Lo et al., 2005) and treatment with these antibodies achieves a more complete blockade of entry into mucosal lymph nodes than is achieved by loss of CD62L function (Arbones et al., 1994; Berlin-Rufenach et al., 1999). CCR7 deficiency resulted in more rapid exit of T cells from mesenteric and peripheral lymph nodes relative to the co-transferred control T cells (Fig. 1A). B cell exit efficiencies were not affected by CCR7 deficiency (Suppl. Fig. 1). As another approach to examine the exit efficiency of CCR7-deficient cells, we generated mixed bone marrow chimeras and measured the frequency of wild-type and CCR7-deficient cells in lymph versus lymph nodes. In mice reconstituted with a mixture of wild-type CD45.1 and wild-type CD45.2 cells, the frequency of the two types of cells in lymph nodes and in lymph were similar (Fig. 1B). In contrast, in mice reconstituted with a mixture of wild-type CD45.1 cells and *Ccr7*$^{-/-}$ CD45.2 cells, there was an enrichment of *Ccr7*$^{-/-}$ cells in lymph compared to lymph nodes (Fig. 1B). The frequency of CD45.1 and CD45.2 B cells in lymph and lymph nodes was similar for both groups (Suppl. Fig. 1). These observations suggest that CCR7-deficient T cells exit into lymph more rapidly than wild-type T cells.

Analysis of *Ccr7*$^{-/-}$ T cell distribution in lymph node sections revealed that the cells were mostly located near HEV and areas positive for the lymphatic marker, LYVE-1, and they were not distributed in the deeper T zone (Fig. 1H) as expected (Förster et al., 1999). Interestingly, the mean $S1P_1$ surface expression was lower on CCR7-deficient T cells than on control T cells present in the same lymph nodes, although some of the CCR7-deficient cells did express normal

amounts of the receptor (Fig. 1C). It seemed likely that the lower expression reflected increased exposure of the mispositioned cells to S1P because lymphocyte $S1P_1$ is highly sensitive to down-modulation by S1P (Lo et al., 2005; Schwab et al., 2005).

In an attempt to distinguish whether CCR7-deficiency led to more rapid egress because the T cells were more poised to respond to exit promoting signals or because of their inability to distribute through the T zone and away from possible exit sites, we performed two further types of experiments. First, we asked whether *Ccr7*$^{+/-}$ T cells showed evidence of more rapid egress. Flow cytometric analysis confirmed that *Ccr7*$^{+/-}$ T cells have about half the normal amount of CCR7 (Fig. 1D). Using the adoptive transfer followed by entry blockade procedure, *Ccr7*$^{+/-}$ cells showed a trend toward more rapid egress compared to co-transferred wild-type control cells at 8 hours (not shown) and a significant difference after 14 hours (Fig. 1E). Flow cytometric analysis of lymph and lymph node cells from recipient mice that had not been antibody treated showed a greater frequency of *Ccr7*$^{+/-}$ cells in lymph compared to lymph nodes (Fig. 1F). However, in contrast to the *Ccr7*$^{-/-}$ cells, *Ccr7*$^{+/-}$ cells were distributed throughout the lymph node T zone (Fig. 1H) and they had normal amounts of surface $S1P_1$ (Fig. 1C).

In the second type of experiment, we asked whether over-expression of CCR7 could lead to increased retention of cells in lymph nodes. Circulating peripheral T cells from transgenic mice over-expressing CCR7 under the control of a CD4 minigene (Kwan and Killeen, 2004) have ~2 fold more CCR7 on their

surface (Fig. 1D). Transgenic T cells were co-transferred with control T cells into wild-type mice, equilibrated for one day and further cell entry into lymph nodes was inhibited using integrin neutralizing antibodies. Co-transferred cells were enumerated at the time of entry blockade (t = 0 h) and twelve hours later (t = 12 h). A greater fraction of the CCR7 transgenic T cells were retained in mesenteric and peripheral lymph nodes compared to co-transferred control cells over the 12 hours (Fig. 1G). In contrast to CCR7-deficient T cells, the transgenic T cells were distributed throughout the T zone (Fig. 1H) and they exhibited normal surface $S1P_1$ levels (Fig. 1C). Taken together these results indicate that CCR7 promotes T cell retention in lymph nodes and they suggest the involvement of mechanisms other than regulation of gross distribution within the T zone.

CCR7 deficiency promotes T cell egress in FTY720 treated hosts

To examine the possibility that $S1P_1$ acts to promote lymphocyte egress by overcoming lymphoid tissue retention signals, we set out to test whether CCR7 deficiency reduced the dependence of T cells on $S1P_1$ for undergoing egress. As a component of these studies, we further examined the relationship between FTY720 treatment and modulation of lymphocyte $S1P_1$ function. We treated mice for the same short (4-5 hr) time period and with doses corresponding to the range previously used to define the IC_{50} for egress inhibition by the active FTY720 enantiomer (Rosen et al., 2003). Comparison of the effect of FTY720 on T cell $S1P_1$ surface expression and on cell numbers in lymph and blood showed that there was a strong positive correlation (Fig. 2A, B). Notably, FTY720 at 0.05mg/kg caused approximately 50% $S1P_1$ down-modulation,

achieving amounts similar to cells with heterozygous deficiency in *Edg1*, the gene encoding $S1P_1$ (Fig. 2C), and the treatment led to a similar decrease in T cell numbers in lymph and blood to the reductions observed in *Edg1*$^{+/-}$ mice (Fig. 2B and D). These findings provide support for the conclusion that FTY720-induced down-modulation of lymphocyte $S1P_1$ contributes to the mechanism by which this drug reduces lymphocyte egress.

To test whether $S1P_1$ functioned in part to overcome CCR7 mediated retention, wild-type mice were reconstituted with a mixture of wild-type and CCR7-deficient bone marrow cells and they were then treated with FTY720 to modulate $S1P_1$ function. The mice were also treated with integrin-blocking antibodies to ensure that any differences observed were not due to differences in lymph node entry. As expected, FTY720 strongly inhibited lymph node egress and reduced numbers of cells were present in lymph after one day of combined FTY720 treatment and entry blockade (Fig. 3A). However, amongst the T cells continuing to appear in lymph at this time there was a strong increase in the representation of CCR7-deficient cells (Fig. 3A, B). In contrast, there was no change in the proportion of CCR7-deficient B cells appearing in the lymph (Fig. 3A). To correct for differences in the extent of reconstitution by the CD45.1 and CD45.2 bone marrow cells in individual animals, the frequency of each T cell type in the lymph was normalized by that in the lymph nodes of the same animals and this confirmed that there was a significant overrepresentation of CCR7-deficient cells in lymph of FTY720 treated animals (Fig. 3C). Thus, although total numbers of T cells were reduced in the lymph of all the animals after FTY720

treatment, the CCR7-deficient cells were reduced by only 60-70% versus more than 90% for wild-type cells. The treatments did not cause detectable changes in lymph node T cell numbers over the period of these experiments. Taken together, these findings support a model where $S1P_1$ functions within T cells in part to overcome CCR7-mediated retention.

Pertussis Toxin treatment promotes egress of $S1P_1$-deficient cells

Though reproducible, CCR7-deficiency led to only a partial recovery in T cell egress into lymph of FTY720 treated mice. We next asked whether additional Gαi-coupled receptors were involved in retention by using a pertussis toxin (PTX) pulse-loading procedure that allows cell entry into lymph nodes during the first 2-3 hours after transfer, prior to Gαi inactivation by the PTX enzyme (Lo et al., 2005; Okada and Cyster, 2007) (see Methods). Cells pulse-loaded with PTX or the non-enzymatic (Oligomer B) subunit as control were co-transferred into saline or FTY720 treated recipients and after 3 hours further entry of cells into lymph nodes was blocked by treatment with integrin neutralizing antibodies. Cell numbers were measured at 0 and 21 h after integrin blockade. Using this approach we found that Gαi inactivation facilitated release of T cells (Fig. 4A) and B cells (Suppl. Fig. 1) from lymph nodes of FTY720 treated mice. When compared to saline treated hosts, a similar fraction of cells was released suggesting that the main effect of FTY720 had been overcome by lymphocyte PTX-treatment (Fig. 4A). The low numbers of cells that seed recipient lymphoid tissues in this adoptive transfer approach made lymph measurements difficult but amongst the small number of cells in lymph of FTY720 treated animals there was

a strong bias in favor of PTX treated cells (Suppl. Fig. 2). Analysis of blood samples showed that greater numbers of transferred PTX-treated than control-treated cells were present at 0 h, perhaps due to inefficient entry into tissues (Fig. 4A). At 21 h the number PTX-treated cells in the blood of saline- and FTY720-treated recipients were comparable, consistent with similar extents of egress (Fig. 4A).

We then asked whether Gαi inhibition would restore egress competence to T cells that lacked $S1P_1$ expression. T cells from the thymus of $S1P_1$-deficient fetal liver chimeras were pulsed with PTX or oligomer B and then transferred to wild-type mice (Fig. 4B). After allowing 3 hours for cell entry into lymph nodes, further entry was blocked by integrin neutralization. One day later we found that a significant fraction of the PTX-treated but not of the oligomer B-treated T cells had been released from the lymph nodes and increased numbers of cells were detected in blood (Fig. 4C). These observations suggest that when Gαi signaling is inhibited $S1P_1$ in T cells is not essential for egress from lymph nodes, and they support the idea that $S1P_1$ promotes egress at least in part by counteracting retention signals mediated by Gαi-coupled receptors including CCR7.

LYVE-1$^+$ cortical sinusoids and lymphocyte exit

For a direct interplay to occur between $S1P_1$ and CCR7 during egress we speculated that both signals needed to be encountered in a common microenvironment. Although T cell egress from lymph nodes is usually considered to occur at the medullary sinuses, areas where CCR7 ligand abundance is low, ultrastructural studies have suggested that ‘upstream’ cortical

sinusoids may also be involved (Kelly, 1975; Soderstrom and Stenstrom, 1969). Staining of lymph node sections for the lymphatic marker LYVE-1 (Jackson et al., 2001) revealed numerous positive cells in the macrophage-rich medullary region, as expected, and also identified LYVE-1^+ sinusoids in the T zone or paracortex, often in areas adjacent to HEV and proximal to B cell follicles (Fig. 5A and B). LYVE-1 staining of sinusoids in the paracortical region has recently been reported (Hirakawa et al., 2005; Prevo et al., 2004). In some lymph node cross-sections, LYVE-1^+ sinusoids could be observed extending from the T cell rich paracortical region to medullary areas (Fig. 5A). These paracortical structures typically contained numerous T cells and B cells (Fig. 5C). These combined observations lead us to categorize the LYVE-1^+ structures in the T zone as being identical to the cortical sinusoids described in ultrastructural studies (Belisle and Sainte-Marie, 1981; Compton and Raviola, 1985; Kelly, 1975; Kowala and Schoefl, 1986; Soderstrom and Stenstrom, 1969). In contrast to their rich lymphocyte content, the cortical sinusoids contained few dendritic cells (Fig. 5C). Because dendritic cells undergo only very limited egress from lymphoid organs, their deficiency in the cortical sinusoids is consistent with these structures playing a role in lymphocyte egress.

Cortical sinusoids were located in areas rich in the CCR7 ligand, CCL21 (Fig. 5D). It is currently not possible to stain for the $S1P_1$ ligand, S1P. We therefore sought an alternative approach to determine whether $S1P_1$ function was required at the level of the cortical sinusoid and tested the impact of $S1P_1$ deficiency on the appearance of transferred T cells in cortical sinusoids.

Thymocytes from *Edg1*$^{-/-}$ or *Edg1*$^{+/+}$ fetal liver chimeras were transferred, together with allelically marked control cells, into wild-type recipients. One day later, few *Edg1*$^{-/-}$ cells could be detected within LYVE-1^{+} cortical sinusoids whereas similar numbers of *Edg1*$^{+/+}$ cells were observed in cortical sinusoids compared to T cells co-transferred as internal controls (Fig. 5E and Suppl. Fig. 3A and B). Enumeration of wild-type and S1P$_1$-deficient T cells within sinusoids and in a 30 µm thick region surrounding each structure confirmed that S1P$_1$-deficient cell accumulation inside sinusoids was strongly inhibited (Fig. 5F). The ratio of S1P$_1$-deficient to wild-type cells in the immediate vicinity of the sinusoid (0.73±0.63, Fig. 5F) was little different than the ratio in the central T zone (0.65±0.16, n=13). A similar frozen tissue analysis of T cell distribution within the medullary region could not be performed because the high density of LYVE-1 staining did not allow sinus and non-sinus areas to be distinguished. These findings indicate T cells require S1P$_1$ for entry into or accumulation within LYVE-1^{+} cortical sinusoids but that the receptor is not critical for T cells to approach these structures.

We next asked whether CCR7 antagonized appearance of cells within sinusoids using *Ccr7*$^{+/-}$ cells because they enter lymph nodes with similar efficiency to wild-type cells and distribute through the T zone (Fig. 1H). *Ccr7*$^{+/-}$ T cells were found at a higher frequency inside LYVE-1^{+} sinusoids than in the immediately surrounding regions (Fig. 5G and Suppl. Fig. 3). In control experiments allelically distinguishable wild-type populations were equally represented in sinusoids and in the surrounding regions (Fig. 5G). These

observations indicate that CCR7 antagonizes T cell entry into or accumulation inside LYVE-1^+ sinusoids and they suggest that this effect cannot be explained solely by influences of CCR7 on cell proximity to these structures.

To further test for a direct interplay between CCR7 and $S1P_1$, we tested the ability of CCL21 to retain cells against an S1P gradient in transwell migration assays. In the absence of CCL21, naïve T cells migrated to the lower chamber in response to S1P (Fig. 5H). However, when CCL21 was included with the cells in the upper chamber at 1 μg/ml, a concentration within the range estimated to exist in lymph node T zones (Luther et al., 2002), wild-type T cells could no longer migrate to S1P in the lower chamber (Fig. 5H). This retention-effect was fully dependent on CCR7 expression by the T cells (Fig. 5H). These results are consistent with the possibility that there is a direct interplay between lymphocyte CCR7 and $S1P_1$ at the level of entry into or retention within LYVE-1^+ cortical sinusoids.

T cells that have divided multiple times become $S1P_1^{hi}$ and have reduced CCR7 function

Our findings identified an antagonistic relationship between CCR7 and $S1P_1$ in determining the egress of naïve T cells. Activated T cells initially downregulate $S1P_1$ transcription and are retained in the responding lymph node and by day three the daughter cells begin recovering $S1P_1$ transcripts and S1P responsiveness (Matloubian et al., 2004). Activated T cells are also known to downregulate CCR7 function (Ansel et al., 1999; Hardtke et al., 2005). Here we examined the relationship between the extent of cell division and the reciprocal

changes in $S1P_1$ and CCR7 function following *in vivo* activation. We found that at day 3 of the OTII T cell response to ovalbumin (OVA) in adjuvant, $S1P_1$ was re-expressed on a large fraction of the T cells that had undergone four or more divisions (Fig. 6A). Chemotaxis assays showed that recovery of $S1P_1$ function was most prominent in cells that had divided four or more times (Fig. 6B). Reciprocally, CCR7 was downregulated in the activated cells as assessed by surface staining (Fig. 6C) and by transcript abundance (Fig. 6D), with the down-modulation being most substantial in the cells that had divided multiple times. CCL21 chemotactic responses were most strongly reduced in cells that had divided more than four times (Fig. 6E). These observations suggest that once antigen-engaged T cells have undergone four or more divisions, many of the cells acquire an $S1P_1^{hi}$ phenotype and are functionally CCR7 low, a combination of changes that most likely reduces the propensity of the cells to migrate further within the T zone and instead favors prompt egress. Consistent with this proposal, there was an enrichment for T cells that had undergone more than 4 divisions amongst the divided T cells in blood and lymph compared to draining lymph node (Fig. 6F).

Discussion

The above findings extend the functions of CCR7 beyond promoting cell entry, compartmentalization and motility within lymphoid organs to demonstrate that it also has a role in favoring lymphocyte retention within these tissues. The requirement for $S1P_1$ in lymphocyte egress is partially relieved by removing

CCR7 and more fully relieved by globally inhibiting lymphocyte Gαi. These findings support a model, elaborated upon below, where $S1P_1$ promotes lymph node egress at least in part by overcoming Gi-mediated retention signals. LYVE-1^+ cortical sinusoids are shown to be a site of interplay between $S1P_1$ and CCR7 responses, with $S1P_1$ promoting T cell accumulation inside sinusoids and CCR7 countering this activity. We also find that inhibition of lymphocyte Gαi largely overcomes the egress inhibitory effects of systemic FTY720 treatment, supporting the conclusion that FTY720 inhibits egress via effects on the lymphocyte. Coincident $S1P_1$ upregulation and CCR7 downregulation occurs in activated T cells that have undergone several cell divisions and might be a mechanism to favor rapid egress of newly generated effector cells.

This study provides new insight regarding both anatomical and signaling aspects of T cell egress from lymph nodes. To integrate these findings and provide a framework for further discussion it is helpful to consider a provisional model for the early events leading to egress. During 'random migration' over stromal cells in the T zone (Bajenoff et al., 2006), T cells are likely to encounter LYVE-1^+ egress structures. In this location they are suggested to be exposed to overlapping distributions of S1P and CCR7 ligand and possibly other retention factors. Each cell then makes a choice to respond to one or the other cue based on the dominance of the signal. Such decision making has been observed for neutrophils responding to overlapping cues in vitro (Foxman et al., 1999; Heit et al., 2002). The molecular basis for this type of interplay remains to be fully defined but seems likely to depend on the ability of a dominantly signaling

receptor to compete successfully for cytoskeletal elements that are needed for achieving cell polarity and movement. Factors that influence which signal is dominant may include: the spatial orientation in which the cell encounters the two cues; the lag in recovery of full $S1P_1$ surface expression following lymph node entry (Lo et al., 2005); possible partial desensitization of CCR7 by ligand (Kohout et al., 2004); changes in receptor abundance or function due to T cell activation status (Ansel et al., 1999; Hardtke et al., 2005; Matloubian et al., 2004); and changes in ligand abundance due to immune response status of the lymph node (Mueller et al., 2007). Partial reductions in receptor (or ligand) expression would be expected to have strong effects on the outcome of this cellular decision-making and the finding that $S1P_1$ and CCR7 hemizygosity strongly influences egress efficiency is consistent with the model. The ability of small (2-3 fold) changes in receptor abundance to cause repositioning of cells in competing chemoattractant gradients has been observed in other systems (Reif et al., 2002). In cases where the CCR7 signal dominates, the cell is suggested to continue random migration in the T zone. In cases where the $S1P_1$ signal dominates, the cell is suggested to localize within the LYVE-1^+ sinusoid. Removal of both S1P and retention receptor responsiveness, for example by PTX treatment, would eliminate the competitive signaling and allow cell localization inside sinusoids by the remaining 'random' motility. The behavior of cells within cortical sinusoids has not yet been characterized but – as suggested long ago (Soderstrom and Stenstrom, 1969; Kelly, 1975) – there may be a general tendency for sinusoidal cells to move toward medullary sinuses and the

efferent lymph. It is recognized that some alternatives to this model are not excluded by current findings and it will be important in future studies to further characterize the dynamics of $S1P_1$-dependent events during T cell egress using real-time imaging procedures.

Cortical sinusoids were possibly first identified by Soderstrom and Stenston in their description of 'mud streams' of lymphocytes in the paracortical (T cell) zone that had a higher density of cells than the average parenchyma and that sometimes appeared as sinus-like structures continuous with the medullary sinus system (Soderstrom and Stenstrom, 1969). Further studies described paracortical sinuses near HEV and connecting with medullary sinuses that were suggested to allow cells to move from the T zone to the medulla (Belisle and Sainte-Marie, 1981; Kelly, 1975). Using LYVE-1 to identify these structures, we found that accumulation of lymphocytes within them was $S1P_1$ dependent and that they contained few dendritic cells, consistent with their playing a role in lymphocyte egress. These experiments also provided functional evidence that S1P is available within or nearby cortical sinusoids. The cell types responsible for generating lymph S1P have not yet been determined though they were shown to be radiation resistant (Pappu et al., 2007) and may correspond to LYVE-1^+ cells.

Our studies do not address to what extent egress occurs via cortical sinusoids versus other sites. In ultrastructural studies, cells can be observed traversing the wall of cortical sinusoids and medullary sinuses (Compton and Raviola, 1985; Heath and Spalding, 1987; Nicander et al., 1991). In addition, treatment with pharmacological $S1P_1$ agonists has been suggested to reduce the

entry of lymphocytes from the medulla into medullary sinuses (Mandala et al., 2002; Sanna et al., 2006; Wei et al., 2005). Moreover, our finding that transferred CCR7-deficient cells are enriched near LYVE-1^+ medullary regions and have down-modulated $S1P_1$ is consistent with cells encountering S1P in this region even before exiting the tissue. Future studies will need to address the extent to which the $S1P_1$-dependent egress step occurs via cortical sinusoids, medullary sinuses and subcapsular sinuses. In addition to locally counteracting $S1P_1$ responses, CCR7 may favor retention over egress by promoting T cell migration and dispersal within the T zone and away from egress sites. In this regard it should be noted that the nature and distribution of the additional Gαi-coupled receptor ligands that are implicated by our studies as promoting T cell retention in lymph nodes is not known. It seems possible that competition between $S1P_1$ and these additional retention systems occurs in other regions of the lymph node such as the medulla.

The ability of T cells to exit lymph nodes even when their Gai signaling is inhibited suggests that the lymph node egress structures may be unusually permissive for cell entry and consistent with this notion, imaging of lymph node medullary sinuses has suggested the presence of portals that may function as egress hotspots (Wei et al., 2005). Ultrastructural studies have suggested that cortical sinusoids may also have openings (Belisle and Sainte-Marie, 1981; He, 1985; Soderstrom and Stenstrom, 1969) though this has been debated (Heath and Spalding, 1987; Nicander et al., 1991). PTX strongly inhibits egress from the thymus indicating that there are unique requirements for egress from this organ,

perhaps reflecting a less penetrable endothelial barrier (reviewed in Cyster, 2005). Although PTX-treatment substantially rescued the egress capability of S1P1-deficient cells, treated cells did not egress from lymph nodes with wild-type efficiency, as noted in a previous study (Lo et al., 2005). Further work will be needed to determine whether the reduction in egress of PTX-treated cells is due to their reduced motility (Huang et al., 2007; Okada and Cyster, 2006) or whether it indicates an additional contribution of $S1P_1$ beyond overcoming Gi-retention signals, such as helping guide cells into regions rich in egress structures. Although we did not observe an effect of $S1P_1$-deficiency on T cell localization near cortical sinusoids, it is possible that T cell migration toward these structures can be promoted by many signals, including $S1P_1$. Moreover, our studies have not examined $S1P_1$-deficient T cell migration into or within the medulla. Consistent with a possible role of this type, treatment with $S1P_1$ agonists altered T cell motility in the medulla but not the T zone (Wei et al., 2005).

Based on the ability of FTY720 to cause marked down-modulation and functional inhibition of $S1P_1$ on lymphocytes, FTY720 was suggested to inhibit lymphocyte egress by causing functional antagonism of $S1P_1$ (Cyster, 2005; Matloubian et al., 2004). An alternative mechanism that has been proposed is agonist action on stromal cells at medullary egress sites to cause closing of egress portals (Sanna et al., 2006; Wei et al., 2005). In our current experiments we observe a tight relationship between the extent of $S1P_1$ surface down-modulation induced by FTY720 on lymph node T cells and the extent of egress inhibition as measured by loss of cells from lymph and blood. A similar potency of

FTY720 in causing downregulation of $S1P_1$ on human lymphocytes was recently reported in an in vitro study (Maeda et al., 2007). Importantly, we find close similarity in the reduction in egress caused by $S1P_1$ heterozygosity as caused by the dose of FTY720 that induces a ~50% decrease in lymphocyte $S1P_1$ abundance. These findings favor the conclusion that FTY720 inhibits egress by functional antagonism of lymphocyte $S1P_1$. In further agreement with this mode of action is our finding that PTX treatment of the lymphocyte largely overcomes the egress-inhibitory effect of FTY720 treatment.

The identification of a role for CCR7 in mediating T cell retention within lymph nodes indicates that reduced CCR7 function can lead to decreased cell numbers in lymphoid organs through accelerated egress as well as reduced entry. For example, the poor ability of CCR7-deficient regulatory T cells to suppress T cell proliferation in lymph nodes may partly reflect inefficient retention of these cells (Schneider et al., 2007). Similarly, the reduced CCL21 expression in lymphoid tissues following certain viral infections (Mueller et al., 2007) might be a mechanism to promote release of T cells into circulation. Finally, our experiments suggest that the regulated egress of newly developing effector cells is controlled not just by down-modulation and re-expression of $S1P_1$ (Lo et al., 2005; Matloubian et al., 2004) but also by the parallel changes in CCR7 function. Early after activation $S1P_1$ transcripts and protein are markedly down-regulated, consistent with the initial retention of activated cells in the responding lymphoid tissue. However, after approximately four divisions, many of the cells not only upregulate $S1P_1$ but they also lose much of their CCR7 function, a combination

of changes that may help ensure these newly generated effector cells do not further scan the lymph node T zone for antigen but exit rapidly into circulation to travel to the site of infection.

Experimental Procedures

Mice and Adoptive Cell Transfer. CD45.2 C57BL/6 (B6) and CD45.1 B6 mice were from the National Cancer Institutes or a colony maintained at the University of California, San Francisco. *Ccr7*$^{-/-}$ mice (Forster et al., 1999) and CCR7 Tg mice (Kwan and Killeen, 2004) were 10 generations backcrossed to B6. OTII B6 mice were as described (Barnden et al., 1998). $S1P_1$ deficient thymocytes were generated by reconstitution of irradiated B6-CD45.1 mice with *Edg1*$^{-/-}$ E12.5 fetal liver cells as described (Matloubian et al., 2004). *Ccr7* wild-type and *Ccr7*$^{-/-}$ mixed bone marrow chimeras were made as described (Reif et al., 2002) using 50:50 mixtures of wild-type CD45.1 and littermate control or *Ccr7*$^{-/-}$ CD45.2 bone marrow. In some experiments, ~$2x10^7$ cells per mL were labeled with 3.3 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen/Molecular Probes) or 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR, Invitrogen/Molecular Probes; (10 μM) in RPMI1640 containing 2% FCS for 20 minutes at 37°C then washed by spinning through a layer of fetal calf serum. For OTII transfers, mice that received $2x10^7$ CFSE labeled spleen and lymph node cells 1 day before were immunized s.c. with 200 μg OVA (Sigma) in CFA (Sigma) as previously described (Matloubian et al., 2004). Lymph collection was performed as described (Matloubian et al., 2004). Briefly, under a stereomicroscope, lymph was drawn from the cysterna chyli using a fine borosilicate glass microcapillary pipette (Sutter Instrument). Cell numbers determined by flow cytometry were divided by the volume of collected lymph to

determine the concentration. Protocols were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco.

Antibodies and Treatments. The anti-αL (clone M17/4, rat IgG2a) hybridoma was from American Type Culture Collection and the anti-α4 (clone PS/2, rat IgG2b) hybridoma was kindly provided by David Erle (University of California, San Francisco). Antibodies were administered intraperitoneally at 100 μg per mouse in PBS. Cells were treated with 10 ng/mL of Oligomer B or PTX at 37°C for 10 min, washed twice in warm RPMI, 2% FCS, and 10 mM Hepes, and then transferred to recipient mice. For generation of a LYVE-1 specific mAb, DT569 cells (Ba/F3 cells stably transfected with mouse IL-2 plasmid) were transfected with mouse LYVE-1 inserted into the pEF vector with sequence encoding the preprolactin leader sequence and Flag epitope inserted in place of the codons for the first 24 amino acids of LYVE-1. Rats were immunized with transfected cells at RnD Systems and the resultant hybridoma supernatants were screened for reactivity to LYVE-1 transfected cells by flow cytometry. Mab 22 stained LYVE-1 transfected cells but not control DT569 cells transfected with Flag-tagged CCR7. This antibody is now available from RnD systems as clone 223322. Anti-CCR7 monoclonal antibody was obtained from BioLegend.

Chemotaxis Assays. Cells were washed in RPMI1640 with 0.5% fatty acid free BSA several times, resensitized for 30 minutes at 37°C then tested for transmigration across uncoated 5 μm transwell filters (Corning Costar Corp.) for 3 h to sphingosine-1-phosphate (S1P) (Sigma-Aldrich) or CCL21 (R&D Systems)

in the bottom chamber as described (Reif et al., 2002). FTY720 was from a custom synthesis performed by SRI International (Menlo Park, CA) and was administered i.p. in saline.

Immunohistochemical and Flow Cytometric Analysis. 7 μm cryostat sections were fixed and stained as described (Reif et al., 2002). CFSE labeled cells were visualized in sections with alkaline-phosphatase conjugated anti-fluorescein antibodies (Roche). Congenic transferred lymphocytes were visualized by staining with biotinylated antibodies to CD45.1 (clone A20) or CD45.2 (Clone 104). For visualization of LYVE-1^+ structures in sections, either unconjugated or biotinylated Mab22 was used. Lymphocyte preparations were stained with various fluorochrome conjugated antibodies purchased from BD Pharmingen or anti-$S1P_1$ as described (Lo et al., 2005) and data were acquired on an FACS LSRII (Beckton Dickinson) and analyzed with FlowJo software (Treestar).

References

Allende, M.L., Dreier, J.L., Mandala, S., and Proia, R.L. (2004). Expression of the sphingosine-1-phosphate receptor, S1P1, on T-cells controls thymic emigration. J. Biol. Chem. *279*, 15396-15401.

Ansel, K.M., McHeyzer-Williams, L.J., Ngo, V.N., McHeyzer-Williams, M.G., and Cyster, J.G. (1999). In vivo activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J. Exp. Med. *190*, 1123-1134.

Arbones, M.L., Ord, D.C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D.J., and Tedder, T.F. (1994). Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity *1*, 247-260.

Arnold, C.N., Butcher, E.C., and Campbell, D.J. (2004). Antigen-Specific Lymphocyte Sequestration in Lymphoid Organs: Lack of Essential Roles for alpha(L) and alpha(4) Integrin-Dependent Adhesion or Galpha(i) Protein-Coupled Receptor Signaling. J. Immunol. *173*, 866-873.

Bajenoff, M., Egen, J.G., Koo, L.Y., Laugier, J.P., Brau, F., Glaichenhaus, N., and Germain, R.N. (2006). Stromal cell networks regulate lymphocyte entry, migration and territoriality in lymph nodes. Immunity *doi:10.1016/j.immuni.2006.10.011*.

Barnden, M.J., Allison, J., Heath, W.R., and Carbone, F.R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol. Cell Biol. *76*, 34-40.

Belisle, C., and Sainte-Marie, G. (1981). Tridimensional study of the deep cortex of the rat lymph node. III. Morphology of the deep cortex units. Anat. Rec. *199*, 213-226.

Berlin-Rufenach, C., Otto, F., Mathies, M., Westermann, J., Owen, M.J., Hamann, A., and Hogg, N. (1999). Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1- deficient mice. J. Exp. Med. *189*, 1467-1478.

Compton, C.C., and Raviola, E. (1985). Structure of the sinus-lining cells in the popliteal lymph node of the rabbit. Anat. Rec. *212*, 408-423.

Cyster, J.G. (2005). Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu. Rev. Immunol. *23*, 127-159.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23-33.

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23-33.

Foxman, E.F., Kunkel, E.J., and Butcher, E.C. (1999). Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation. J. Cell Biol. *147*, 577-588.

Hardtke, S., Ohl, L., and Forster, R. (2005). Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. Blood *106*, 1924-1931.

He, Y. (1985). Scanning electron microscope studies of the rat mesenteric lymph node with special reference to high-endothelial venules and hitherto unknown lymphatic labyrinth. Arch. Histol. Jpn. *48*, 1-15.

Heath, T.J., and Spalding, H.J. (1987). Pathways of lymph flow to and from the medulla of lymph nodes in sheep. J. Anat. *155*, 177-188.

Heit, B., Tavener, S., Raharjo, E., and Kubes, P. (2002). An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J. Cell Biol. *159*, 91-102.

Hirakawa, S., Kodama, S., Kunstfeld, R., Kajiya, K., Brown, L.F., and Detmar, M. (2005). VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. Journal of Experimental Medicine *201*, 1089-1099.

Huang, J.H., Cardenas-Navia, L.I., Caldwell, C.C., Plumb, T.J., Radu, C.G., Rocha, P.N., Wilder, T., Bromberg, J.S., Cronstein, B.N., Sitkovsky, M.*, et al.* (2007). Requirements for T lymphocyte migration in explanted lymph nodes. J. Immunol. *178*, 7747-7755.

Jackson, D.G., Prevo, R., Clasper, S., and Banerji, S. (2001). LYVE-1, the lymphatic system and tumor lymphangiogenesis. Trends Immunol. *22*, 317-321.

Kelly, R.H. (1975). Functional anatomy of lymph nodes. I. The paracortical cords. Int. Arch. Allergy Appl. Immunol. *48*, 836-849.

Kohout, T.A., Nicholas, S.L., Perry, S.J., Reinhart, G., Junger, S., and Struthers, R.S. (2004). Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. J. Biol. Chem. *279*, 23214-23222.

Kowala, M.C., and Schoefl, G.I. (1986). The popliteal lymph node of the mouse: internal architecture, vascular distribution and lymphatic supply. J. Anat. *148*, 25-46.

Kwan, J., and Killeen, N. (2004). CCR7 directs the migration of thymocytes into the thymic medulla. J. Immunol. *172*, 3999-4007.

Lo, C.G., Xu, Y., Proia, R.L., and Cyster, J.G. (2005). Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J. Exp. Med. *201*, 291-301.

Luther, S.A., Bidgol, A., Hargreaves, D.C., Schmidt, A., Xu, Y., Paniyadi, J., Matloubian, M., and Cyster, J.G. (2002). Differing Activities of Homeostatic Chemokines CCL19, CCL21, and CXCL12 in Lymphocyte and Dendritic Cell Recruitment and Lymphoid Neogenesis. J. Immunol. *169*, 424-433.

Maeda, Y., Matsuyuki, H., Shimano, K., Kataoka, H., Sugahara, K., and Chiba, K. (2007). Migration of CD4 T Cells and Dendritic Cells toward Sphingosine 1-Phosphate (S1P) Is Mediated by Different Receptor Subtypes: S1P Regulates the Functions of Murine Mature Dendritic Cells via S1P Receptor Type 3. J. Immunol. *178*, 3437-3446.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C*., et al.* (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science *296*, 346-349.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

Mueller, S.N., Hosiawa-Meagher, K.A., Konieczny, B.T., Sullivan, B.M., Bachmann, M.F., Locksley, R.M., Ahmed, R., and Matloubian, M. (2007).

Regulation of homeostatic chemokine expression and cell trafficking during immune responses. Science *317*, 670-674.

Nicander, L., Nafstad, P., Landsverk, T., and Engebretsen, R.H. (1991). A study of modified lymphatics in the deep cortex of ruminant lymph nodes. J. Anat. *178*, 203-212.

Okada, T., and Cyster, J.G. (2006). B cell migration and interactions in the early phase of antibody responses. Curr. Opin. Immunol. *18*, 278-285.

Okada, T., and Cyster, J.G. (2007). CC Chemokine Receptor 7 Contributes to Gi-Dependent T Cell Motility in the Lymph Node. J. Immunol. *178*, 2973-2978.

Pappu, R., Schwab, S.R., Cornelissen, I., Pereira, J.P., Regard, J.B., Xu, Y., Camerer, E., Zheng, Y.W., Huang, Y., Cyster, J.G., and Coughlin, S.R. (2007). Promotion of Lymphocyte Egress into Blood and Lymph by Distinct Sources of Sphingosine-1-Phosphate. Science *316*, 295-298.

Prevo, R., Banerji, S., Ni, J., and Jackson, D.G. (2004). Rapid plasma membrane-endosomal trafficking of the lymph node sinus and high endothelial venule scavenger receptor/homing receptor stabilin-1 (FEEL-1/CLEVER-1). J. Biol. Chem. *279*, 52580-52592.

Reif, K., Ekland, E.H., Ohl, L., Nakano, H., Lipp, M., Forster, R., and Cyster, J.G. (2002). Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. Nature *416*, 94-99.

Rosen, H., Alfonso, C., Surh, C.D., and McHeyzer-Williams, M.G. (2003). Rapid induction of medullary thymocyte phenotypic maturation and egress inhibition by nanomolar sphingosine 1-phosphate receptor agonist. Proc. Natl. Acad. Sci. U S A *100*, 10907-10912.

Rosen, H., Sanna, M.G., Cahalan, S.M., and Gonzalez-Cabrera, P.J. (2007). Tipping the gatekeeper: S1P regulation of endothelial barrier function. Trends Immunol. *28*, 102-107.

Sanna, M.G., Wang, S.K., Gonzalez-Cabrera, P.J., Don, A., Marsolais, D., Matheu, M.P., Wei, S.H., Parker, I., Jo, E., Cheng, W.C.*, et al.* (2006). Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P(1) antagonist in vivo. Nat. Chem. Biol. *2*, 434-441.

Schneider, M.A., Meingassner, J.G., Lipp, M., Moore, H.D., and Rot, A. (2007). CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. J. Exp. Med. *204*, 735-745.

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J.G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science *309*, 1735-1739.

Soderstrom, N., and Stenstrom, A. (1969). Outflow paths of cells from the lymph node parenchyma to the efferent lymphatics--observations in thin section histology. Scand. J. Haematol. *6*, 186-196.

von Andrian, U.H., and Mempel, T.R. (2003). Homing and cellular traffic in lymph nodes. Nat. Rev. Immunol. *3*, 867-878.

Wei, S.H., Rosen, H., Matheu, M.P., Sanna, M.G., Wang, S.K., Jo, E., Wong, C.H., Parker, I., and Cahalan, M.D. (2005). Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. Nat. Immunol. *6*, 1228-1235.

Worbs, T., Mempel, T.R., Bolter, J., von Andrian, U.H., and Forster, R. (2007). CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. J. Exp. Med. *204*, 489-495.

Acknowledgments

We are grateful to Reinhold Forster, Martin Lipp, Richard Proia and Nigel Killeen for mice, Olivia Lam for assistance with the mouse colony, Joao Pereira and Ying Xu for technical assistance and Steve Rosen and members of the Cyster lab for comments on the manuscript. T.H.M.P. is supported by the Boyer Program in the Biochemical Sciences and the UCSF Medical Scientist Training Program. J.G.C. is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by grants from the NIH. The authors declare they have no financial conflicts of interest.

Figure Legends

Figure 1. CCR7 affects T cell egress rates from lymph nodes. (A) Wild-type and *Ccr7*$^{-/-}$ spleen cells were co-transferred to wild-type recipients and after 24 h, the mice were treated with integrin-blocking antibodies for 8 h. The data shown indicate the fraction of T cells remaining in recipient mesenteric (m) and peripheral (p) lymph nodes at 8 h compared to 0 hr. (B) Irradiated mice were reconstituted using mixtures of CD45.2^{+} *Ccr7*$^{+/+}$ or *Ccr7*$^{-/-}$ bone marrow and CD45.1^{+} WT bone marrow and the representation of CD45.2^{+} cells in peripheral and mesenteric lymph nodes (LN) and in lymph (LYM) was determined. (C) Flow cytometric analysis of $S1P_1$ on *Ccr7*$^{-/-}$ and *Ccr7*$^{+/+}$ CD4 T cells from lymph nodes of mixed-bone marrow chimeras (left panel) and *Ccr7*$^{+/-}$ (middle panel) or *Ccr7-Tg* (right panel) CD4^{+} T cells together with co-transferred control cells. Shaded histograms show staining with control antibody. (D) Flow cytometric data showing CCR7 expression using anti-CCR7 mAb on *Ccr7*$^{+/-}$ and littermate control (upper panel) or *Ccr7-Tg* and littermate control (lower panel) lymph node CD4^{+} T cells. Shaded histograms show staining of *Ccr7*$^{-/-}$ cells. (E and F) Transfers and integrin blockade were performed as in A. In E the fraction of cells remaining after 14 h in the indicated lymph nodes was determined. F shows the ratio of transferred *Ccr7*$^{+/-}$ and *Ccr7*$^{+/+}$ T cells in peripheral and mesenteric lymph nodes (LN) and in lymph (LYM) at 0 h. (G) Wild-type control and *Ccr7-Tg* cells were transferred to wild-type recipients and the mice were treated as in A. The data shown indicate the fraction of T cells remaining in the indicated lymph nodes at 12 h compared to 0 h of entry blockade. In A-B and E-G, bars indicate mean and

points indicate data from individual mice. (H) Distribution of WT, *Ccr7*$^{-/-}$, *Ccr7*$^{+/-}$ and *Ccr7-Tg* cells within peripheral lymph nodes. CFSE labeled T cells were transferred into wild-type recipients and one day later lymph node sections were stained to detect transferred cells (blue) and LYVE-1^{+} sinusoids (brown). Objective magnification, 5x.

Figure 2. Dose sensitivity of FTY720-mediated S1P$_1$ modulation and induction of lymphopenia. (A) FACS analysis of S1P$_1$ on lymph node CD4^{+} T cells 4.5 h after in vivo treatment with the indicated amounts of FTY720. (B) Fraction of CD4^{+} T cells remaining in blood (BLD) and lymph (LYM) 4.5 h after treatment with the indicated amounts of FTY720 compared to saline treated controls. (C) FACS analysis of S1P$_1$ on wild-type littermate control and *Edg1*$^{+/-}$ lymph node CD4^{+} T cells. (D) Number of CD4^{+} T cells in blood and lymph of wild-type and *Edg1*$^{+/-}$ mice. Data are representative of at least 3 mice for each condition. Bars represent means and dots individual mice.

Figure 3. FTY720-mediated inhibition of T cell egress is partially CCR7 dependent. (A) Flow cytometric analysis of CD45.2^{+} *Ccr7*$^{-/-}$ and CD45.1^{+} WT T and B cells in mixed bone marrow chimeras treated for 24 h with saline or FTY720 and with integrin blocking antibodies for 20 h. Numbers indicate percent cells in the indicated gates out of total T or B cells. The means (±SD) of lymphocyte numbers in the samples were as follows: saline lymph=6.3x10^{3}/ul ($\pm$ 4.5x10^{3}), n=8; FTY720 lymph=7.5x10^{2}/ul ($\pm$ 9.7x10^{2}), n=9; saline LN=1.3x10^{7} ($\pm$ 4.0x10^{6}), n=8; FTY720 LN=1.6x10^{7} ($\pm$ 8.1x10^{6}), n=9. (B) Frequency of CD45.2^{+} T

cells in the peripheral LN, mesenteric LN and lymph of mice that had been reconstituted as in A (*Ccr7*$^{-/-}$ mixed chimera) or with a mixture of CD45.2^{+} *Ccr7*$^{+/+}$ and CD45.1^{+} WT bone marrow as a further control (*Ccr7*$^{+/+}$ mixed chimera). (C) Same data as in B plotted as the ratio of each type of T cell in lymph versus peripheral lymph nodes for each animal. Bars in B and C represent mean and points individual animals and the data are pooled from three experiments.

Figure 4. PTX treatment facilitates egress of FTY720 exposed and S1P$_1$ deficient cells. (A) Egress of PTX or oligomer B treated T cells from lymph nodes of FTY720 treated mice. Splenocytes were treated with PTX or with Oligomer B (Oligo-B) as a control and co-transferred into hosts pre-treated with saline or FTY720 4 h earlier. Three hours after transfer, the mice were treated with integrin-blocking antibodies. The number of remaining cells in lymph nodes 21 h after "entry blockade" was determined. Numbers shown are normalized for the number of input splenocytes. (B and C) PTX restores egress of S1P$_1$-deficient lymphocytes. (B) Diagram of transfer experiment. (C) Number of transferred Oligomer B or PTX treated *Edg1*$^{-/-}$ or *Edg1*$^{+/+}$ T cells in peripheral lymph nodes and blood at 0 h and 27 h of integrin blockade. Numbers shown are normalized for the number of CD62L-high, single-positive input thymocytes. Bars represent mean and points individual animals.

Figure 5. S1P$_1$ deficient cells are found less frequently within cortical sinusoids. (A) Serial sections of lymph node stained for LYVE-1 (brown) and IgD (blue) or CD11b (blue) showing LYVE-1 expressing structures extending

from cortical areas into the macrophage-rich medullary region. (B) Proximity of cortical LYVE-1^+ structures (blue) to PNAd expressing HEV (brown). (C) LYVE-1^+ cortical structures (brown) contain B cells (IgD, blue) and T cells (CD3, blue) but are relatively devoid of dendritic cells (CD11c, blue). (D) CCL21 expression (blue) in wild-type lymph nodes with respect to LYVE-1 (brown). (E) Effect of $S1P_1$-deficiency on T cell appearance in cortical sinusoids. CD45.2^+ thymocytes from wild-type or $S1P_1$ deficient fetal liver chimeras were labeled with CFSE and each population was co-transferred with wild-type (CD45.1^+) thymocytes into B6 (CD45.2^+) mice. Thirty-six hours after transfer, recipient lymph nodes were sectioned and stained to detect the fetal liver chimera-derived (CSFE$^+$) T cells (blue) and the co-transferred wild-type cells (CD45.1, red) and for LYVE-1 (E). Objective magnifications: (A) 5x; (B-D) 10x; (E) 20x. (F and G) Enumeration of (F) *Edg1*$^{+/+}$ and *Edg1*$^{-/-}$ cells or (G) *Ccr7*$^{+/+}$ and *Ccr7*$^{+/-}$ cells and co-transferred wild-type control T cells inside LYVE-1^+ sinusoid structures (In) and in a 30 μm thick area surrounding each sinusoid (Out). Data are plotted as ratio of wild-type, *Edg1*$^{-/-}$ or *Ccr7*$^{+/-}$ cells to co-transferred control cells in each region. Each dot represents data from a single sinusoid cross-section and bars represent the means. (H) Transwell migration of *Ccr7*$^{+/+}$ or *Ccr7*$^{-/-}$ CD4$^+$ T cells in response to the indicated concentrations of S1P. Cells were added to the upper wells in the absence or presence of 1 μg/ml CCL21 as indicated. Data are representative of three experiments.

Figure 6. T cells that have divided multiple times upregulate $S1P_1$ and downregulate CCR7. Mice receiving CFSE-labeled OTII T cells were immunized s.c. with OVA in CFA or left unimmunized and draining lymph node cells were analyzed at day 3. (A) Flow cytometric analysis of $S1P_1$ versus CFSE on OTII T cells. The line indicates the baseline determined by staining cells with a control antibody. (B) Transwell migration assay showing relationship between cell division (Div) number and recovery of S1P responsiveness. OTII T cells that had divided 0-3 times are shown by dashed lines, 4-8 times by thin solid lines and endogenous $CD4^+$ T cells by a thick solid line. (C) Flow cytometric analysis of CCR7 versus CFSE on OTII T cells. (D) RT-QPCR analysis of CCR7 on sorted OTII T cells that had divided the indicated number of times based on CFSE dilution. (E) Transwell migration assay showing relationship between cell division number and reduced CCL21 responsiveness. Bars show mean±sd (n=4). (F) Overlay of CFSE profile in lymph node versus blood and lymph, showing enrichment for highly divided cells in circulation. Data are representative of at least 3 experiments.

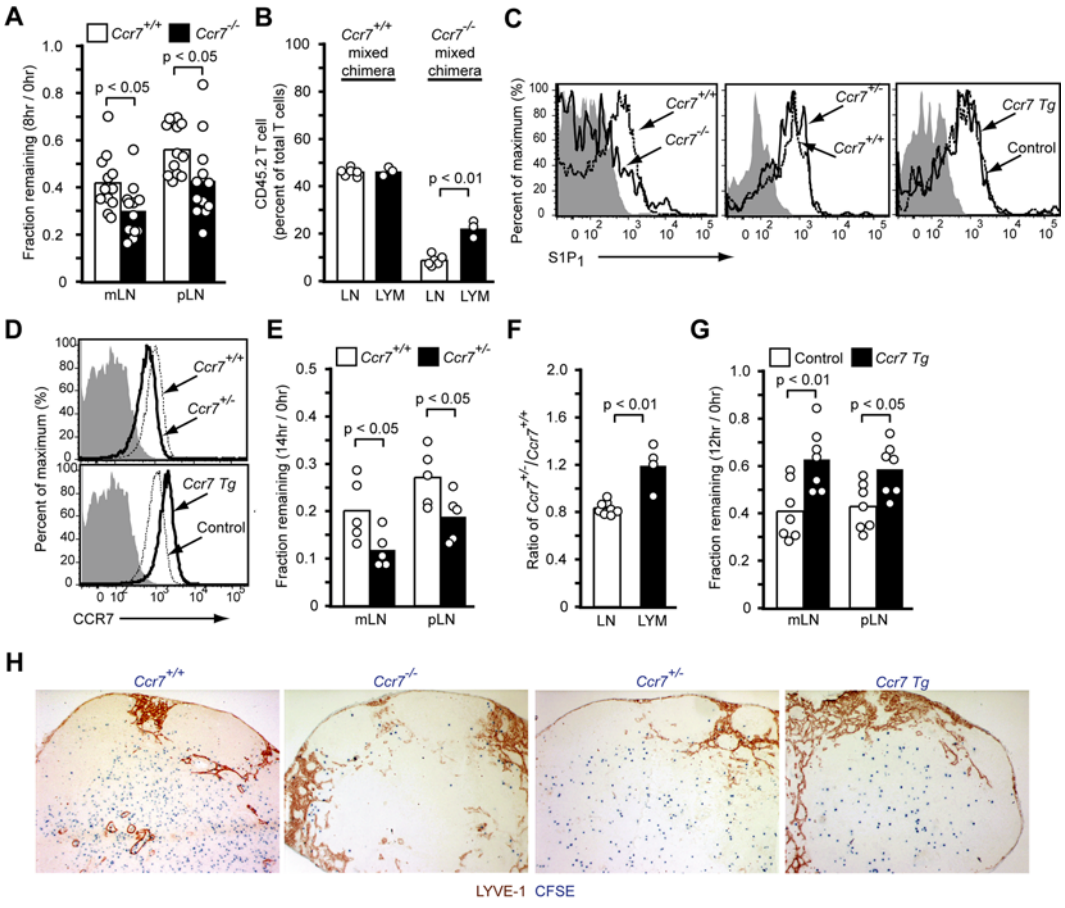
Supplemental Figures.

Supplemental Figure 1. Effect of CCR7 deficiency and PTX treatment on B cell egress from lymph nodes. (A and B) Wild-type and CCR7-deficient B cells have similar egress efficiency. (A) Transfers and integrin blockade were performed as in Figure 1A, and the fraction of cells remaining after 8 h in the indicated lymph nodes was determined. (B) Irradiated mice were reconstituted using mixtures of CD45.2^{+} *Ccr7*$^{+/+}$ or *Ccr7*$^{-/-}$ bone marrow and CD45.1^{+} WT bone marrow as described in Figure 1B and the representation of CD45.2^{+} cells in peripheral and mesenteric lymph nodes (LN) and in lymph (LYM) was determined. (C) Egress of PTX or oligomer B treated B cells from lymph nodes of FTY720 treated mice. Data are from the same animals shown in Figure 3B. The number of remaining cells in lymph nodes 21 h after "entry blockade" was determined. Numbers showed are normalized for the number of input splenocytes.

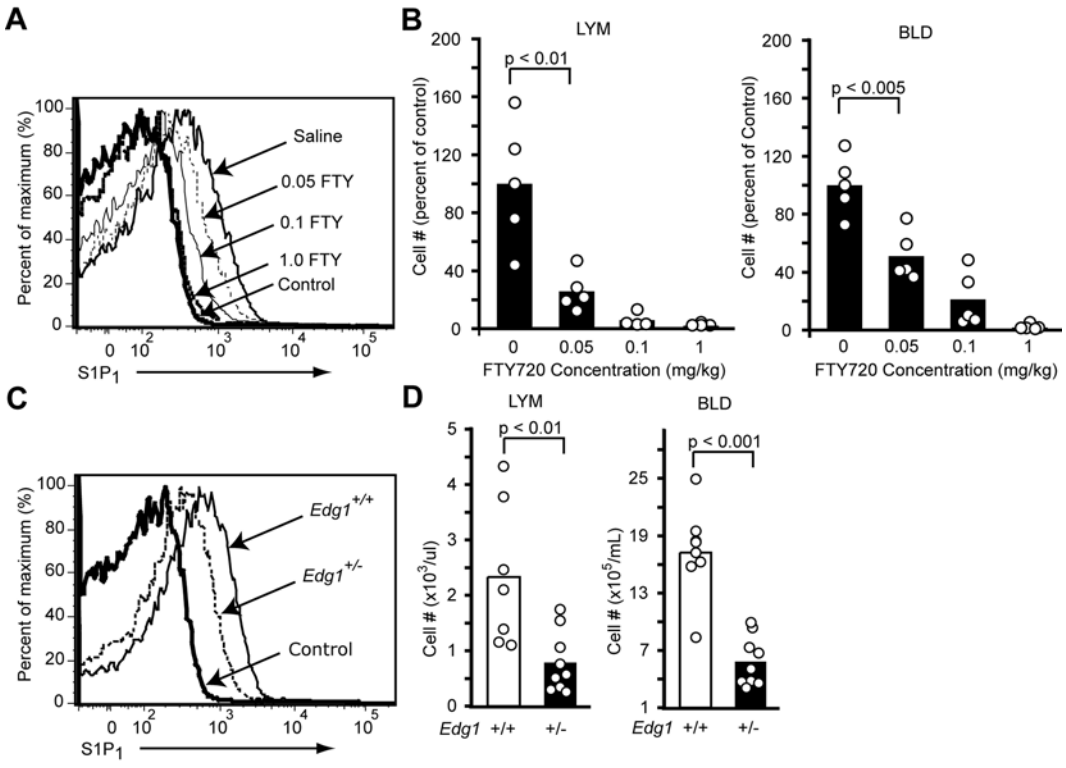
Supplemental Figure 2. PTX treatment facilitates egress of T lymphocytes into lymph of FTY720 treated mice. (A) Splenocytes were labeled with CFSE or CMTMR and treated with PTX or Oligo-B, respectively, and transferred into hosts pre-treated with FTY720 4 h earlier. Profiles show flow cytometric analysis of transferred cells in the lymph and lymph nodes of hosts 22 h after transfer. Numbers represent the percentages of transferred T cells in the indicated gates out of total T cells. Samples from three individual mice are shown. (B) Splenocytes were treated with PTX or Oligo-B, transferred into hosts pre-treated with saline or FTY720 and treated 3 h later with integrin-blocking antibodies as in figure 4. The frequency of transferred cells in the lymph 21 h after "entry

blockade" was determined. Numbers shown are percent transferred T cells out of total lymph samples. Typical lymph collections were 1-3 µl. Bars represent means and dots individual mice. The mean (±SD) number of transferred T cells per µl of lymph were: 9.3±5.2 Oligo-B treated cells and 3.0±2.0 PTX treated cells in control (saline) treated hosts; 1.3±1.2 Oligo-B treated cells and 6.4±3.1 PTX treated cells in FTY720 treated hosts.

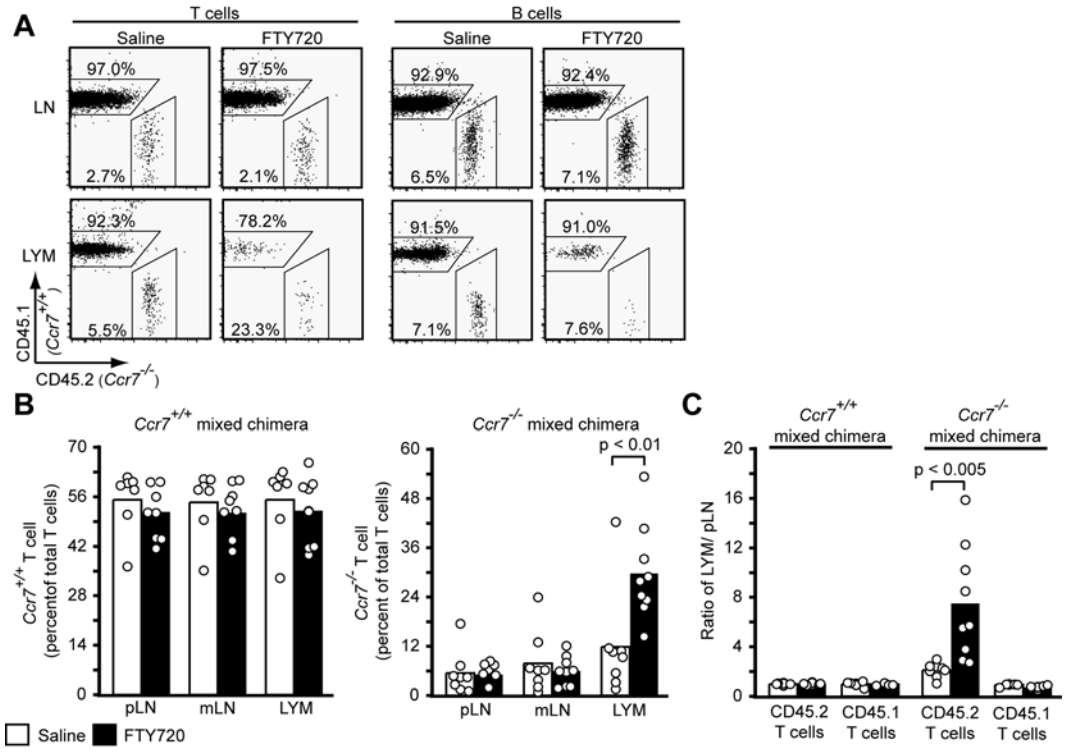
Supplemental Figure 3. Effect of $S1P_1$-deficiency or reduced CCR7 signaling on T cell appearance in cortical sinusoids. (A and B) Further examples of the type of analysis shown in Fig. 5E. $CD45.2^+$ thymocytes from $S1P_1$-deficient (A) or wild-type (B) fetal liver chimeras were labeled with CFSE and each population was co-transferred with wild-type ($CD45.1^+$) thymocytes into B6 ($CD45.2^+$) mice. Thirty-six hours after transfer, recipient lymph nodes were sectioned and stained to detect the fetal liver chimera-derived ($CSFE^+$) T cells (blue) and the co-transferred wild-type cells (CD45.1, red) and for LYVE-1. Data are representative of peripheral lymph nodes from at least three recipient mice of each type. (C and D) Examples of the histological analysis used to generate the data shown in Fig. 5G. CFSE labeled *Ccr7*$^{+/+}$ (C) or *Ccr7*$^{+/-}$ (D) cells were co-transferred with $CD45.1^+$ control cells into B6 mice. After 36 h immunohistochemistry was performed as described in A and B. White dashed line defines a 30um thick area surrounding the LYVE-1^+ structure. Scale bar, 30 µm. Objective magnification: 20x.



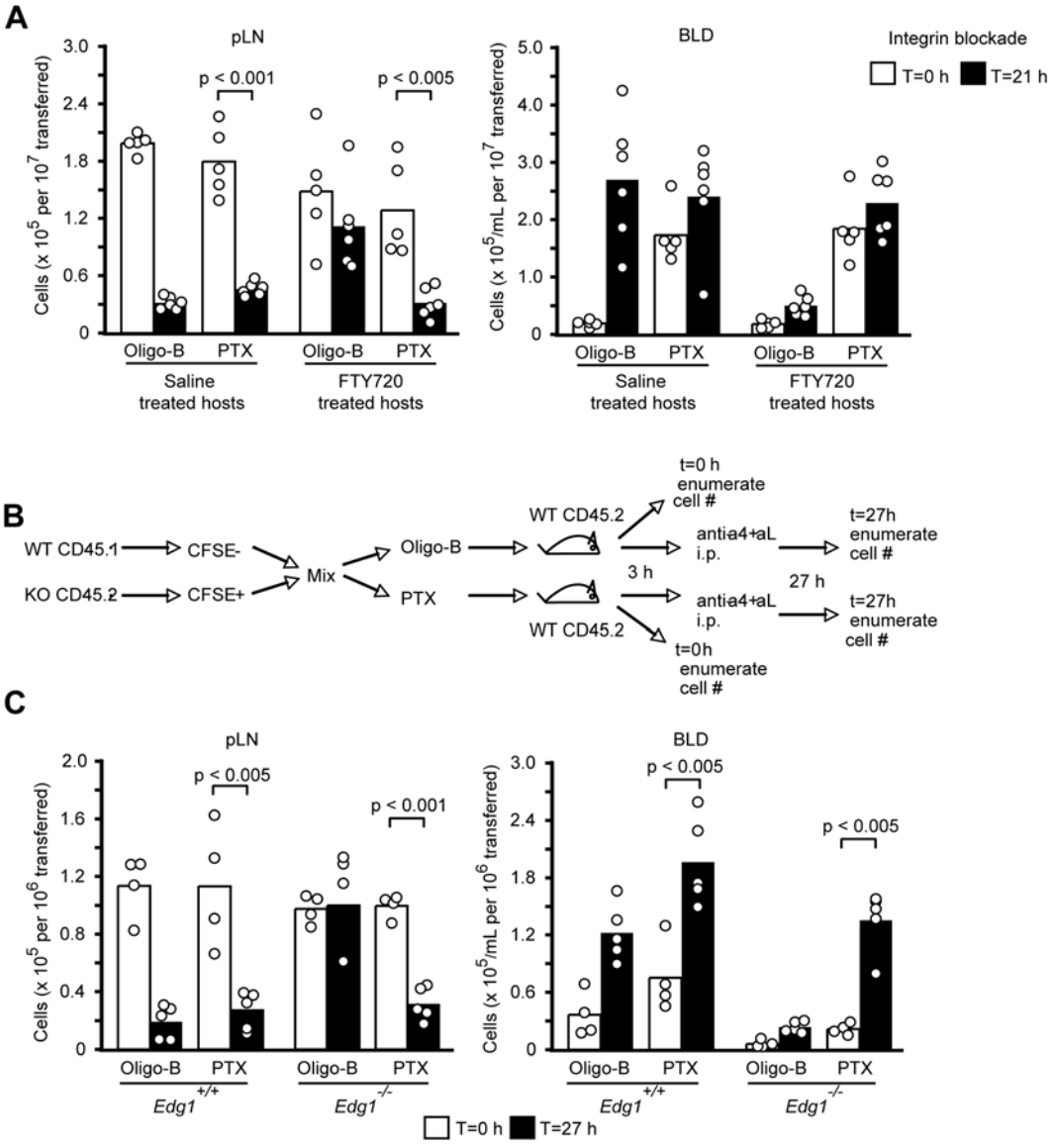
Chapter 2, Figure 1



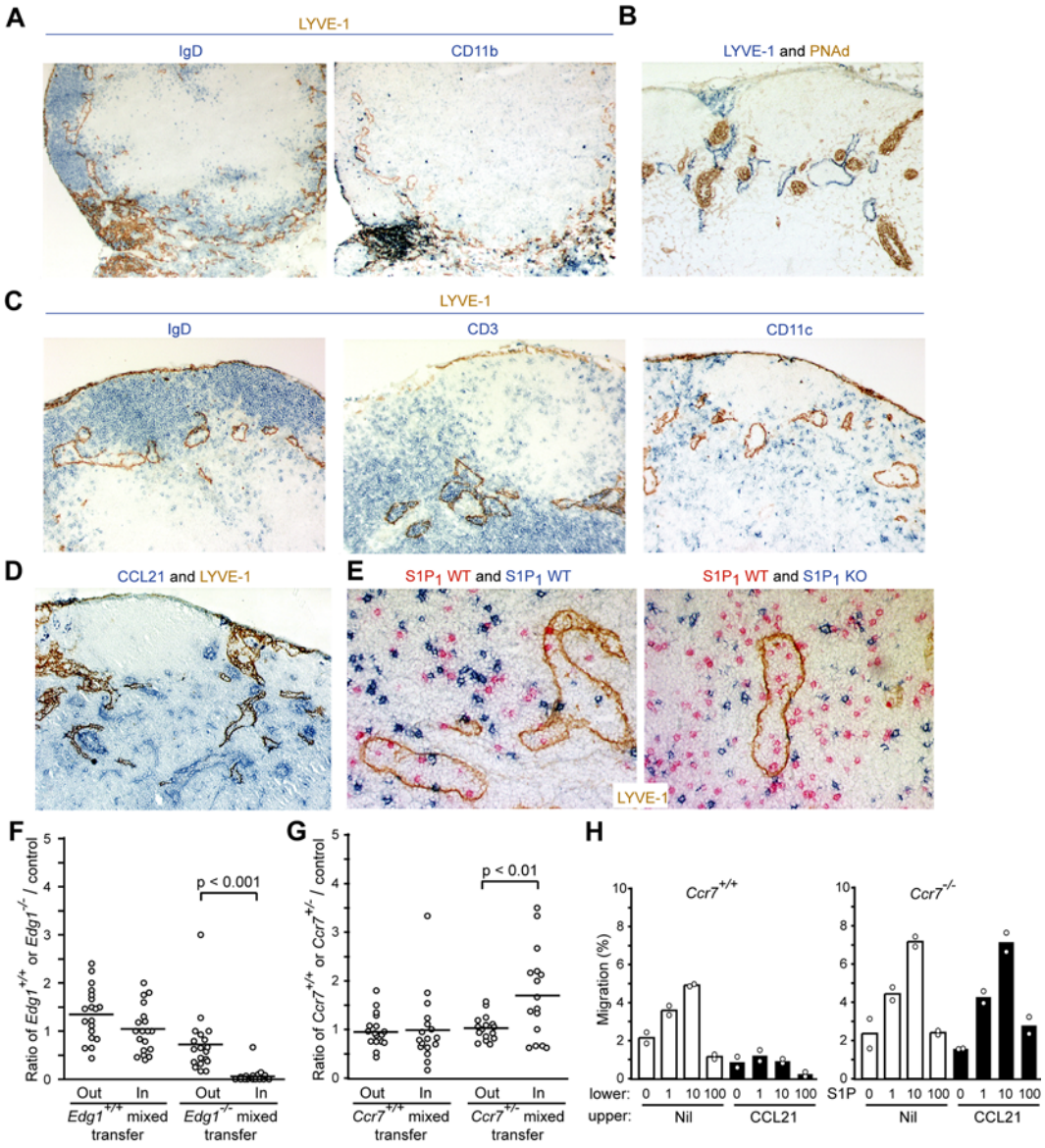
Chapter 2, Figure 2



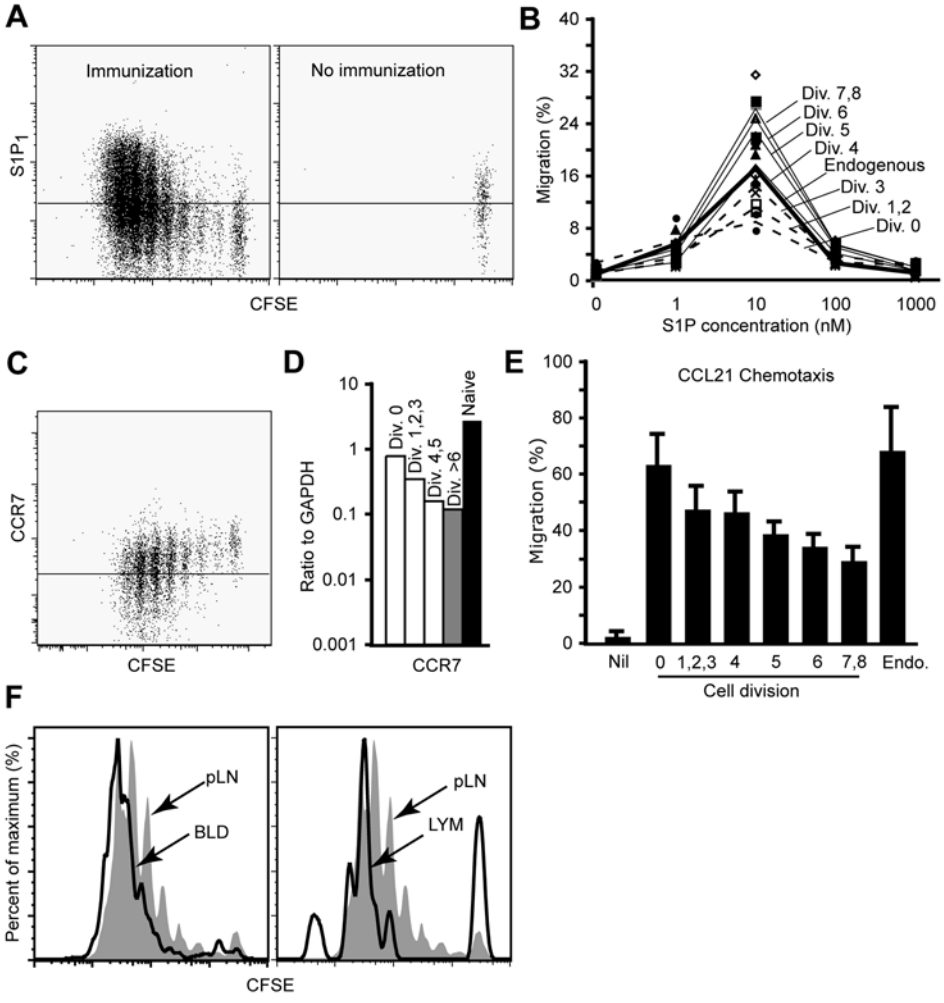
Chapter 2, Figure 3



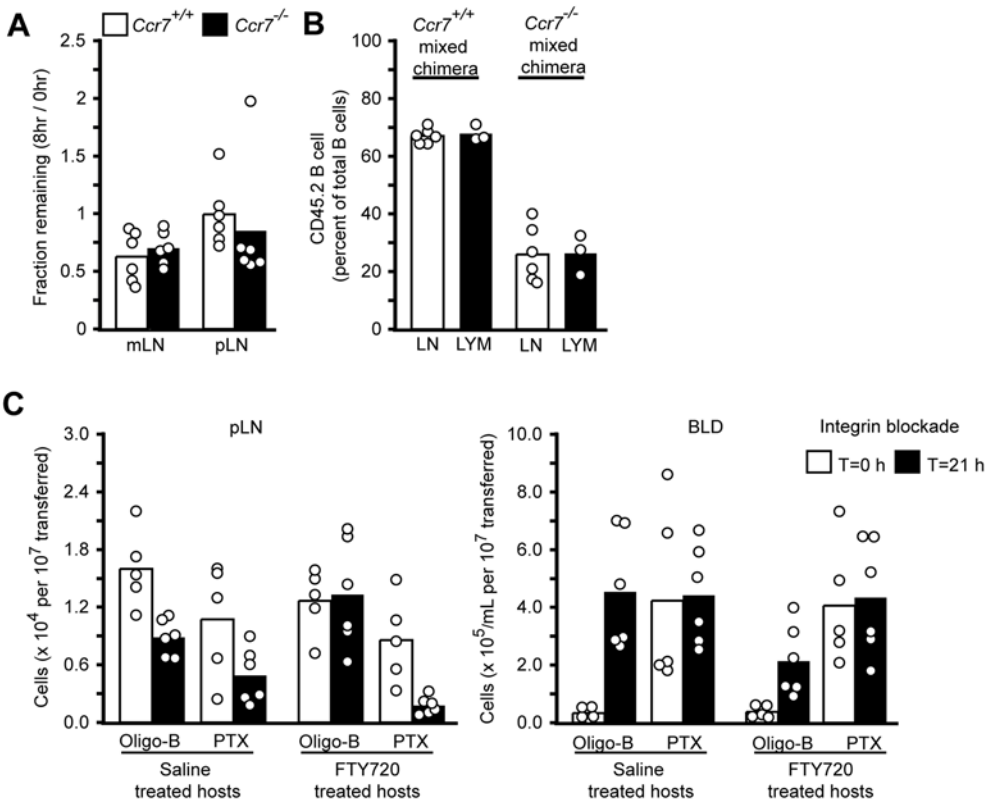
Chapter 2, Figure 4



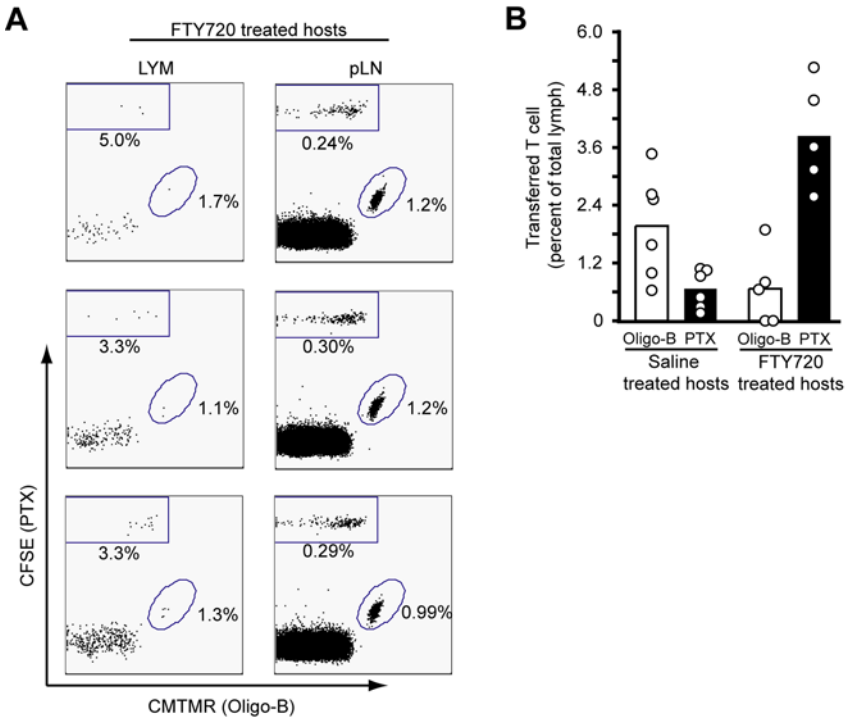
Chapter 2, Figure 5



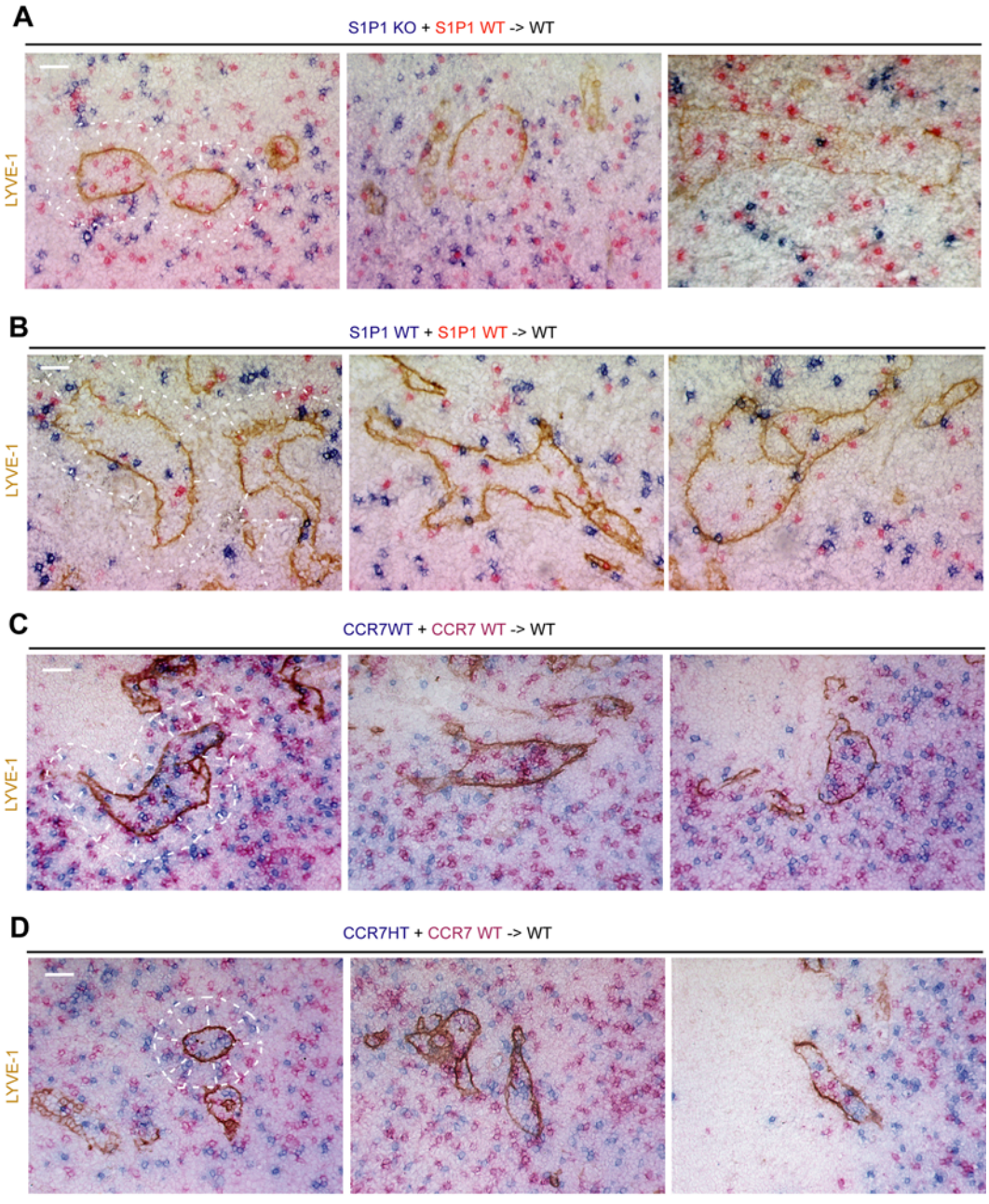
Chapter 2, Figure 6



Chapter 2, Figure S1



Chapter 2, Figure S2



Chapter 2, Figure S3

CHAPTER 3

Lymphatic endothelial cell-derived S1P is required for lymphocyte egress from lymph nodes and for lymphatic patterning

Summary

Lymphocyte egress from lymph nodes is dependent on sphingosine-1-phosphate (S1P), but the cellular source of this S1P is not defined. We generated mice that expressed *Cre* from the lymphatic vessel endothelial hyaluronan receptor-1 (*Lyve-1*) locus and that showed efficient recombination of LoxP-flanked genes in lymphatic endothelium. We report that mice with *Lyve-1* CRE-mediated ablation of Sphingosine kinase *(Sphk)-1* and lacking *Sphk2* have a loss of S1P in lymph while maintaining normal plasma S1P. In lymphatic *Sphk*-deficient mice, lymphocyte egress from lymph nodes and Peyer's patches is blocked. Treatment with pertussis toxin to overcome Gαi-mediated retention signals restores lymphocyte egress. Furthermore, in the absence of lymphatic *Sphks*, the architecture of lymphatic vessels in non-lymphoid tissue is altered. Our data establish lymphatic endothelial cells as the *in vivo* source of lymph S1P required for lymphocyte egress from lymph nodes and Peyer's patches and demonstrate a role for S1P in lymphatic vessel maturation.

Introduction:

After entry and a period of intra-nodal transit, T and B cells egress from lymph nodes into the lymph in a process that requires lymphocyte-intrinsic S1P1, a G protein-coupled receptor (GPCR) (Matloubian et al., 2004). Recent studies demonstrated that S1P1 acts to overcome lymphocyte retention mediated by CCR7 and other Gαi-coupled signals to promote egress from lymph nodes (Pham et al., 2008). S1P1 is needed for lymphocytes to migrate into exit structures, the LYVE1+ cortical sinuses of the lymph nodes, within which they may be captured by lymphatic flow and transported to the efferent lymph (Grigorova et al., 2009; Sinha et al., 2009). S1P is critical for lymphocyte egress from the lymphoid tissues (Pappu et al., 2007). S1P is normally low in the lymphoid tissue and abundant in blood and lymph, and disruption of this S1P gradient results in an egress block (Schwab et al., 2005). However, despite its importance for lymphocyte egress, the cellular source of lymph S1P remains unknown (Schwab and Cyster, 2007).

S1P is a product of sphingolipid metabolism and its cellular production is dependent on sphingosine kinase (Sphk)-1 and -2 (Kono et al., 2008). S1P and the five receptors, S1P1-5, on which it acts, are involved in a wide-range of biological processes (Kono et al., 2008). Yet, not much is known about the *in vivo* sources of extracellular S1P that are relevant for different physiological processes. This is due, in part, to the fact that all cells appear capable of producing S1P intracellularly (Schwab and Cyster, 2007). Recent work has demonstrated that red blood cells are a major source of plasma S1P (Pappu et

al., 2007). This study also found that all lymph S1P and about 5% of plasma S1P were supplied by a distinct, radiation resistant source. Restoration of plasma S1P to normal levels by bone marrow transplantation in *Sphk*-deficient mice established that lymph and plasma S1P are maintained as separate pools. *In vitro* studies have shown that blood vessel endothelial cells (BECs) can act as a source of S1P (Venkataraman et al., 2008). However, it has not been determined whether endothelial cells are an important source of S1P *in vivo*.

Lymphatic endothelial cells (LECs) arise from the venous endothelium during embryonic development around E9.0-9.5, when a subpopulation of endothelial cells of the anterior cardinal vein commit to the lymphatic lineage by turning on *Lyve-1* and *Prox-1* expression (Karpanen and Alitalo, 2008). LYVE-1 is the earliest expressing and one of the most specific and widely used markers for LECs (Karpanen and Alitalo, 2008; Oliver and Srinivasan, 2008).

S1P signaling has a central role in the development of the cardiovascular system (Kono et al., 2008). Mice lacking *Sphk1* and *Sphk2* die *in utero* between E11.5-E13.5 due to vascular defects that result in widespread hemorrhages (Mizugishi et al., 2005). *In vitro*, stimulation of endothelial cells with S1P increases localization of VE-cadherin at cell-cell junctions and induces tubular morphogenesis (Lee et al., 1999). Recently, S1P was demonstrated to promote tubular formation of human dermal LECs *in vitro* and lymphangiogenesis in matrigel *in vivo* (Yoon et al., 2008) and to stimulate angiopoietin-2 exocytosis (Jang et al., 2009). However, whether S1P signaling normally plays a role in the development of the lymphatic system is not known.

In this study, by examining mice that lack *Sphk2* and have *Sphk1* conditionally deleted by a CRE recombinase expressed from the *Lyve-1* locus we established that LECs are the source of lymph S1P. Lymphatic *Sphk*-deficient mice experienced a block of T and B cell egress from lymph nodes and their LYVE-1+ cortical sinuses lacked lymphocytes. Pertussis toxin (PTX) treatment of lymphocytes resulted in their recovery in the lymph and presence in cortical sinuses in these mice, indicating that the sinuses are competent to support lymphocyte egress. Additionally, lymphatic *Sphk*-deficient mice displayed altered initial lymphatic vessel morphology and junctional VE-cadherin patterning in trachea and diaphragm. Our data support the conclusion that LECs produce lymph S1P required for lymphocyte egress, as well as provide evidence for a role of S1P signaling in the normal maturation of the lymphatic system.

Results:

Ablation of lymph S1P by conditional deletion of *Sphk1* in *Sphk2* null mice

In order to achieve ablation of *LoxP* flanked genes in LECs, we generated a knock-in mouse line in which an *EGFP-hCre* transgene preceded by an IRES element was inserted into the 3' untranslated region of the *Lyve-1* gene (Suppl. Fig.1). In preliminary experiments we were unable to detect fluorescence of the EGFP, likely reflecting the low amount of expression of the fusion protein (data not shown) and for simplicity we refer to the knockin mice as *Lyve1-Cre* mice. Experimental mice carried the *Cre* knock-in gene, two *Sphk2* null alleles, and both floxed or a combination of floxed and null *Sphk1* alleles. Mice having at

least one functional *Sphk* allele were used as controls. Flow cytometric analysis of lymphocytes in the lymph of *Lyve-1 Cre+ Sphk 1*$^{f/-\ or\ f}$ *2*$^{-/-}$ mice showed high surface S1P1, as compared to controls, which had undetectable amounts of receptor (Fig. 1A). Lymphocyte S1P1 is highly sensitive to S1P-induced down-modulation and 1nM S1P down-modulates S1P1 on mature thymocytes (Schwab et al., 2005). Thus, the high level of surface S1P1 on lymphocytes in the lymph of lymphatic *Sphk*-deficient mice suggested a marked reduction from the normal 100-300 nM lymph S1P concentration (Pappu et al., 2007). In contrast, lymphocytes from the blood of lymphatic *Sphk*-deficient and control mice both had undetectable surface S1P1 levels (Fig. 1A), consistent with blood S1P being maintained mostly by red blood cells (Pappu et al., 2007). As a second approach to accessing the level of lymph S1P in the *Sphk*-deficient mice, we employed a FLAG-S1P1 down-modulation bioassay that had previously been demonstrated to be a sensitive method for measuring tissue and fluid S1P levels (Pappu et al., 2007; Schwab et al., 2005). Consistent with the flow cytometric analysis, the lymphatic *Sphk*-deficient mice had undetectable amounts of S1P in lymph, indicating levels are at least 50-fold lower than in control mice (Fig. 1B). There was no difference in blood S1P abundance between lymphatic *Sphk*-deficient and control mice (Fig. 1B).

Specificity of *Lyve-1* CRE-mediated gene deletion

To determine the efficiency and specificity of *Lyve-1* CRE-mediated gene deletion *Lyve-1 Cre*+ mice were intercrossed to mice carrying YPF preceded by a

floxed transcriptional stop in the Rosa26 locus (Rosa26YFP reporter mice) (Srinivas et al., 2001) and their offspring were analyzed for YFP expression in LECs and other cell types. By flow cytometric analysis, we identified LECs as $CD45^{-}$ $CD31^{hi}$ gp38 (podoplanin)^{hi} cells (Fig. 2A) (Link et al., 2007). To confirm the identity of these cells as LECs, we further demonstrated that they express high amounts of surface LYVE-1 (Fig. 2A), as compared to $CD45^{-}$ $CD31^{hi}$ $gp38^{lo}$ blood endothelial cells (BECs), $CD45^{-}$ $CD31^{lo}$ $gp38^{hi}$ T-zone reticular cells (TRCs), and other $CD45^{-}$ lymph node stromal cells. When analyzed for reporter expression, more than 90 percent of LECs were YFP-positive (Fig. 2B), indicating efficient CRE-mediated gene deletion in these cells. A varying fraction of BECs was also positive for YFP reporter expression among lymphoid tissues, probably due to differential expression of LYVE-1 in subsets of BECs that has been observed during embryonic development (Gordon et al., 2008). By contrast, less than 10 percent of T zone reticular cells were YFP-positive. In our analyses, we also observed a fraction of lymphocytes and myeloid cells were YFP-positive (data not shown). The reporter gene expression in these cells likely represents partial CRE-mediated deletion of the floxed transcriptional termination element in hematopoieic stem or precursor cells (data not shown).

By QPCR analysis we detected selective expression of *Lyve-1* and *Prox-1* in the LEC population, confirming their lymphatic identity (Fig. 2C). LECs expressed *Sphk1* and *Sphk2* abundantly, compared to total spleen tissue, while making lesser amounts of enzymes that degrade S1P such as S1P lyase, lipid phosphatases, and S1P-phosphatases (Fig. 2C). Notably, there was a striking

difference in the expression of these enzymes in LECs as compared to T zone reticular cells (Fig. 2C), consistent with the idea that S1P availability is tightly compartmentalized. Recently, SPNS2 was implicated as an S1P transporter in zebrafish (Kawahara et al., 2009; Osborne et al., 2008). Transcripts for the murine SPNS2 orthologue were abundantly expressed in LECs (Fig. 2C).

To further establish the efficiency and specificity of CRE-mediated gene deletion in *Lyve-1 Cre* mice, we performed immunofluorescence studies of tissue sections. Consistent with the flow cytometric analysis, immunofluorescence analysis of lymph node tissues obtained from *Lyve-1 Cre* YFP reporter mice that had been lethally irradiated and reconstituted with wild-type YFP-negative bone marrow showed extensive overlap between YFP and LYVE-1 immunoreactivity (Fig. 2D). In particular, the cells lining cortical sinusoids, identified by their LYVE-1 staining and location in the T cell zone, showed YFP expression (Fig. 2D). YFP expression was also variably observed in non-LYVE-1+ endothelial cells, as well as some other cells that are likely radiation-resistant lymphocytes. YFP expression was also observed in LYVE-1+ lymphatic vessels in ear dermis and in small intestine (Suppl. Fig. 2 A & B).

LYVE-1 expressing macrophages are not a significant source of lymph S1P

Besides LECs, macrophages have also been suggested to express LYVE-1 (Jackson, 2004). Although previous bone marrow chimera studies showed a lack of contribution by radiation-sensitive hematopoietic cells to lymph S1P, the extent of macrophage replacement by donor-derived cells was not assessed (Pappu et

al., 2007). To rule out a contribution of LYVE-1+ lymph node macrophages to lymph S1P, we performed bone marrow chimera studies, reconstituting lymphatic *Sphk*-deficient mice with wild-type bone marrow. Using flow cytometric analysis of cells isolated from enzyme-digested lymph nodes, we identified two $CD45^+$ cell populations, $CD11b^{hi}$ $CD11c^{med}$ and $CD11b^{hi}$ $CD11c^{lo}$ subsets, that expressed LYVE-1 (Fig. 3A & B). Previous studies suggested these two gates contain cells of monocyte lineage and a substantial fraction of them express the macrophage markers F4/80 and/or CD169 ((Jakubzick et al., 2008) and (data not shown)). When we examined lymph nodes of irradiated $CD45.2^+$ lymphatic *Sphk*-deficient and control mice that had been reconstituted with congenically marked CD45.1 wild-type bone marrow, we observed that at least 80% to almost 100% of various myeloid cell populations were donor-derived (Fig. 3C). Particularly, the $CD11b^{hi}$ $CD11c^{med}$ and $CD11b^{hi}$ $CD11^{lo}$ subsets exhibited more than 85% and 95% chimerism, respectively (Fig. 3C). In these bone marrow chimeras, lymph S1P was reduced to the same extent observed in non-chimeric *Sphk*-deficient animals, as indicated by the recovery of surface S1P1 of T cells in the lymph (Fig. 3D) and by S1P bioassay analysis (Fig. 3E). Taken together, our data support the conclusion that hematopoietic cells do not make a significant contribution to lymph S1P.

Impaired lymphocyte egress in lymphatic *Sphk*-deficient mice

Consistent with the marked reduction in lymph S1P, T and B lymphocyte egress from lymph nodes were drastically reduced as evident from the low number of

cells in lymph isolated from the cysterna chyle of lymphatic *Sphk*-deficient mice (Fig. 4A). We also detected a similar extent of egress impairment in the bone marrow chimeras discussed in figure 3 (Suppl. Fig. 2C), consistent with the idea that hematopoietic cells make no significant contribution to lymph S1P needed for lymphocyte egress from lymph nodes. There was also a decrease in lymphocyte numbers in the circulation and the spleen (Fig. 4B & C). Since blood S1P levels were normal in lymphatic *Sphk*-deficient mice, lymphocytes presumably could egress from the spleen and traffic to the lymph nodes, where their egress is impaired. Interestingly we did not observe an expansion of the lymphocyte compartment in the lymph nodes, but saw a consistent reduction of lymph node T cell number (Fig. 4D). Concomitantly, there was an accumulation of naïve T cells in the Peyer's patches (Fig. 4E). We observed a larger increase of surface S1P1 on naive T cells recovered from Peyer's patches of lymphatic *Sphk*-deficient mice as compared to the increase exhibited in the lymph nodes, suggesting a more complete loss of S1P availability in the Peyer's patches (Suppl. Fig. 2E).

To further investigate the lymphocyte egress defect in *Lyve-1 Cre*+ *Sphk*-deficient mice, we performed histological analysis on lymph nodes isolated from these animals to examine cell distribution in LYVE-1+ cortical lymphatic sinuses, which are sites of lymphocyte egress from lymph nodes (Grigorova et al., 2009; Pham et al., 2008; Sinha et al., 2009). Consistent with the reduced lymphocyte numbers in the lymph, LYVE-1+ sinuses in lymphatic *Sphk*-deficient lymph nodes contained almost no lymphocytes and appeared collapsed (Fig. 4F). In contrast,

LYVE-1+ sinuses in control lymph nodes were extended with cells (Fig. 4F). A similar emptying of cortical sinuses was seen 6 hours after treatment with the S1P1-modulating and egress inhibiting drug, FTY720 (Rosen and Goetzl, 2005; Schwab and Cyster, 2007), indicating that sinus empyting can occur rapidly when lymphocyte entry is blocked (Supp. Fig. 3). Together our data established a requirement of LEC-derived S1P for localization of lymphocytes in LYVE-1+ cortical sinuses and egress into the lymph. Furthermore, the accumulation of cells in the Peyer's patches of lymphatic *Sphk*-deficient animals correlated with a lack of lymphocytes in nearby intestinal lymphatic sinuses (Fig. 4G) (Azzali, 2003). These findings further establish the involvement of S1P signaling in regulating lymphocyte egress from the Peyer's patches.

Restoration of egress in lymphatic S1P-deficient mice by PTX treatment

We have previously demonstrated that when Gαi-mediated retention signals are blocked, the lymphocyte intrinsic requirement for S1P1 during egress is overcome (Pham et al., 2008). As a further test for whether the lymphocyte egress defect in *Lyve1 Cre+ Sphk*-deficient mice occurs via effects on the lymphocytes, we sought to determine if inhibition of Gαi-mediated retention signaling would restore egress. Wild-type lymphocytes were treated *ex vivo* either with PTX or the non-enzymatic Oligomer-B (OB) subunit of PTX as a control using a pulse-loading procedure that allowed treated cells to continue entry into lymph nodes for 2-3 hours after being transferred into recipient mice, prior to complete inhibition of Gαi (Lo et al., 2005; Pham et al., 2008). After one

day of equilibration, the distribution of transferred cells in host animals was determined. Whereas the lymph frequency of PTX-treated T cells was about one third the frequency of oligomer B-treated cells in the control hosts, their frequency was almost 4 fold higher than that of oligomer B-treated cells in the lymph of lymphatic *Sphk*-deficient hosts (Fig. 5A & B). When their three-fold lower frequency in the lymph node was taken into account, the over-representation of PTX treated T cells in the lymph of *Sphk*-deficient hosts was even more appreciable. These data suggest that while egress of control (oligomer B)-treated T cells was blocked in these animals, PTX-treated cells continue to exit into the lymph, resulting in their over-representation in this compartment. Moreover, when absolute lymph cell numbers were plotted, there was no difference in the numbers of PTX-treated T cells in control and lymphatic *Sphk*-deficient recipients, suggesting that these cells can undergo egress to a similar extent in both types of hosts (Fig. 5C). PTX treatment also restored B cell egress in host animals lacking lymph S1P, implying again that the reduced egress in lymphatic *Sphk*-deficient mice was due to a lack of S1P-mediated signaling in the lymphocytes (Fig. 5A and Suppl. Fig. 4A & B).

As further evidence for restoration of lymphocyte egress in *Sphk*-deficient mice by PTX treatment, we sought to determine whether PTX-treated cells are localized in LYVE-1+ egress structures using immunohistochemical analysis. Purified T-cells or thymocytes were treated with either PTX or oligomer B and co-transferred into control or *Sphk*-deficient hosts as described above. One day later, in the control lymph nodes many oligomer B-treated cells were found in the

LYVE-1+ cortical sinuses (Fig. 5D), whereas very few if any cells localized in the cortical sinuses of the lymphatic *Sphk*-deficient mice (Fig. 5D). By contrast, PTX-treated cells could be identified within the cortical sinuses of control and lymphatic *Sphk*-deficient mice (indicated by arrows) (Fig. 5D and Suppl. Fig. 4C). Overall, many more PTX-treated cells were found inside or overlapping with the cortical sinuses than oligomer B-treated cells in the lymph nodes of lymphatic *Sphk*-deficient mice. PTX-treated cells were found to localize within LYVE-1+ structures in control lymph nodes as well (Fig. 5D, Suppl. Fig. 4C). Taken together, our findings support the conclusion that lymphatic endothelial cell-derived S1P acts on lymphocytes to promote localization of cells within LYVE-1+ sinuses and egress from lymph nodes.

Altered lymphatic vasculature in lymphatic *Sphk*-deficient mice

Because of the established functions of S1P signaling in development of the blood vasculature, we sought to determine if ablation of lymph S1P had an effect on lymphatic vasculature. We examined the lymphatic morphology and architecture of the *Lyve-1 Cre+ Sphk*-deficient mice using the well-established model of trachea and diaphragm afferent lymphatics (Baluk et al., 2007). When visualized by whole-mount staining for LYVE-1, the lymphatic vessels in the trachea of lymphatic *Sphk*-deficient mice appeared tortuous and ragged (Fig. 6 A & B, Suppl. Fig. 5 A & B), with occasional sprouts (indicated by arrowheads in Fig. 6B and also seen in Suppl. Fig. 5A), as compared to the smooth tubular structures of lymphatic vessels in the control mice. To further examine the

lymphatic vessels in the *Lyve-1 Cre+ Sphk*-deficient mice, we stained the diaphragm for VE-cadherin, which is enriched at cell-cell junctions between neighboring LECs (Baluk et al., 2007). While in control mice the characteristic VE-cadherin+ discontinuous junctions at the initial lymphatics were apparent and often consisted of a series of "buttons," the junctions in the affected animals were less defined, usually being made up by fewer buttons (Fig. 6C & D, Suppl. Fig. 5C & D). Consistent with previous studies (Sinha et al., 2009; Yoon et al., 2008), we found that lymph node LECs expressed abundant amounts of *S1p1*, and to a much lesser extent *S1p3* (Suppl. Fig. 5E), suggesting that LECs are capable of transducing signals from extracellular S1P that mediates organization of lymphatic cell-cell junctions.

Discussion

Using a genetic approach, we provide evidence in this study that lymphatic endothelial cells are the source of S1P needed for lymphocyte egress from lymph nodes and Peyer's patches into lymph. Mice in which *Sphk1* and *Sphk2* are deleted in LECs show markedly reduced lymph S1P and reduced lymph node and Peyer's patch egress. Restoration of lymphocyte egress in lymphatic *Sphk*-deficient mice by antagonizing Gαi-mediated retention demonstrated that the egress defect was due to a requirement for S1P to act on the lymphocytes rather than on endothelial egress barriers. In addition, lymphatic *Sphk*-deficient mice exhibit altered morphology and junctional patterning in initial lymphatic vessels in non-lymphoid tissues establishing a role for S1P in maturation of these vessels.

Although all cells are thought to be capable of producing S1P intracellularly, extracellular S1P appears to come predominantly from a restricted set of cells. More than 90% of plasma S1P is derived from hematopoietic cells, with red blood cells being the major source (Pappu et al., 2007). Cells that supply extracellular S1P are likely to have abundant biosynthetic capacity, which is dependent on the amount of cellular S1P-producing and S1P-degrading enzymes (Le Stunff et al., 2004). Red blood cells, for example, express significant amounts of *Sphks*, but have very little S1P lyase or phosphatase activity (Ito et al., 2007; Pappu et al., 2007). Our data showing that LECs express low amounts of several S1P-degrading enzymes but substantial amount of *Sphk1* and *Sphk2* are consistent with LECs being a source of extracellular S1P. Reciprocally, the high expression of lipid phosphatases by T zone reticular cells may contribute to maintaining low interstitial S1P concentrations in the lymph node parenchyma (Schwab et al., 2005). Comparative analysis of global gene expression between LECs and BECs showed proteins involved in transport and secretion are highly represented in LECs, suggesting these cells might be an important source of extracellular molecules (Podgrabinska et al., 2002). Production and directional secretion of S1P and its carrier proteins by LECs may contribute to the compartmentalization of S1P that is required for egress.

Although it is not known whether the LYVE-1+ cells lining lymph node cortical sinuses are identical to lymphatic vessel endothelial cells, our flow cytometric and immunofluorescence microscopy analysis of reporter mice indicate that the *Lyve-1* CRE is active in these cells. Since we previously established that S1P1 is

required for entry into cortical sinuses, we propose that S1P produced by LYVE-1+ cortical sinus lining cells is essential in promoting egress. Further evidence that S1P is made locally by cortical sinus lining cells is the finding that S1P1 on cells adjacent to these structures is modulated from the cell surface (Pham et al., 2008; Sinha et al., 2009). Since cortical sinuses often begin as blind ended structures (Belisle and Sainte-Marie, 1981; He, 1985) and the interstitial fluid in lymph nodes appears to have little S1P (Schwab et al., 2005) this S1P gradient most likely emanates from the LYVE-1+ cortical sinus lining cells themselves.

We have proposed a “competing cues” model for lymphocyte egress from lymph nodes in which S1P in the cortical sinus acts directly on the probing lymphocyte to promote movement away from the retention factors in the tissue parenchyma and into the sinus (Grigorova et al., 2009; Pham et al., 2008). Our finding that PTX treatment of the lymphocytes restores egress in mice lacking lymphatic endothelial cell-derived S1P is consistent with this model for S1P and S1P1 function during lymph node egress. That is, when Gi-mediated retention signaling is removed, the need for the S1P signal is diminished. Moreover, the evidence that S1P is made locally by LYVE-1+ cells that are in areas with abundant CCL21 (Pham et al., 2008) supports the view that lymphocytes undergoing egress encounter retention and egress-promoting signals in close proximity.

The finding that T cell numbers in lymph nodes were reduced in lymphatic *Sphk*-deficient mice despite the reduction in T cell egress from these tissues was unexpected. The increased accumulation of cells in the Peyer’s patches

accounts for some but not all of the missing T cells. One explanation for the reduction may be a requirement for continual recirculation between lymphoid tissues to maintain T cell viability (Link et al., 2007). It is also possible that S1P signaling via one or more of the S1P receptors provides a necessary trophic stimulus, either directly to the T cell or through actions on support cells. Indeed, S1P has been reported to have a pro-survival influence on some cell types (Kennedy et al., 2009). A further possibility is that the role of S1P in lymphatic maturation may affect physical properties of the lymph node. During the preparation of lymph node cell suspensions we noted that the lymph nodes from lymphatic *Sphk*-deficient mice were firmer and more difficult to press through a cell strainer than wild-type lymph nodes, perhaps indicating a greater abundance of collagen in the capsule (unpubl. obs.). Loss of appropriate capsular elasticity (Compton and Raviola, 1985) might lead to a reduced capacity to support normal lymphocyte numbers. It is possible that all of these factors are at play in determining lymph node T cell numbers and a variety of approaches will be needed to study this issue.

Early *in vitro* studies with BECs showed S1P stimulation increased VE-cadherin localization at cell-cell junctions and enhanced adherens-junction assembly (Lee et al., 1999). S1P signaling has also been implicated in promoting tight-junction formation between BECs (Lee et al., 2006; Sanchez et al., 2003) and N-cadherin mediated adhesive interactions between BECs and mural cells (Paik et al., 2004). S1P1 antagonism *in vivo* reduced the integrity of lung blood vessels (Sanna et al., 2006) and increased blood vessel leakiness during shock

(Camerer, 2009), consistent with a role of S1P signaling in tightening endothelial cell junctions. Here, in mice with a loss of lymph S1P, we observe a reduced definition and frequency of VE-cadherin rich, button-like junctions between LECs of the initial lymphatics, suggesting that S1P has a related action on lymphatic endothelium, promoting localization and maintenance of VE-cadherin at cell-cell junctions. The basis for the clustering of VE-cadherin into button-like junctions is not known but it is notable that S1P signaling was suggested to promote a clustered distribution of N-cadherin in BECs (Paik et al., 2004). Recently, S1P was demonstrated to promote tubular formation of human dermal LECs *in vitro* (Yoon et al., 2008). Although normal lymphatic S1P production was not necessary for formation of the lymphatic vascular system, the ragged appearance of initial lymphatics in S1P-deficient mice suggests it also facilitates tube formation *in vivo*. A further *in vitro* study showed S1P stimulated angiopoietin-2 exocytosis in dermal LECs in an S1P1-dependent manner (Jang et al., 2009). Angiopoietin-2 plays an important role in lymphatic development as gene-targeted deletion of *Angpt2* leads to defects in lymphatic architecture and lymphedema (Gale et al., 2002). Since we did not observe lymphedema in lymphatic *Sphk*-deficient mice, factors in addition to S1P must be involved in promoting angiopoietin-2 release. The increased number of ‘spikes’ in the initial lymphatics of *Lyve-1 Cre*+ *Sphk*-deficient mice bears some resemblance to the lymphatic sprouting observed under conditions favoring lymphangiogenesis (Baluk et al., 2005), suggesting lymphatic S1P may help regulate this process under homeostatic conditions.

A major function of the lymphatics is transportation of leukocytes from tissues to the lymph nodes. Despite their altered lymphatic morphology and molecular patterning, the initial lymphatics of *Lyve-1 Cre*+ *Sphk*-deficient mice appear functional at the level of supporting cellular migration in steady state, as the numbers of skin derived-dendritic cells in skin draining lymph nodes of these mice were similar to control mice (unpubl. obs.). Furthermore, our findings that PTX treated lymphocytes are localized in cortical LYVE-1+ sinuses and are able to egress into the lymph of lymphatic Sphk-deficient hosts with the same efficiency as in wild-type hosts suggests the *Sphk*-deficient lymph node lymphatic vessels are structurally capable of supporting lymphocyte egress. The similar lack of cortical sinus lymphocytes in lymph nodes from *Sphk*-deficient mice and 6-hour FTY720 treated mice suggests the apparent 'collapse' or emptying of these sinuses is secondary to the block in lymphocyte entry rather than being a consequence of developmental abnormalities. However, we do not rule out the possibility that cortical sinus endothelial cell junctions are affected by S1P deficiency. Attempts to examine VE-cadherin distribution on cortical sinuses have so far been unsuccessful, perhaps owing to their very thin walls and the dense layer of lymphocytes that surround them. Whether the structural changes seen in the initial lymphatic vessels of lymphatic *Sphk*-deficient mice affect other lymphatic functions such as absorption of macromolecules will be interesting topics for future investigation. Finally, whether the altered lymphatic morphology and molecular patterning seen in the *Lyve-1 Cre*+ *Sphk*-deficient mice is due to direct effects of S1P on LECs or indirect effects on other cells remains to be

determined. S1P1 expression has been observed in LECs in the present study, as well as by others (Jang et al., 2009; Sinha et al., 2009; Yoon et al., 2008) and *in vitro* studies indicate that the receptor is active in these cells. Future work using mice lacking S1P-receptors specifically in LECs may allow further advances in our understanding of how S1P signaling regulates lymphatic development and function.

Experimental Procedures

Mice and Adoptive Cell Transfer. CD45.2 C57BL/6 (B6) and CD45.1 B6 mice were from the National Cancer Institutes or a colony maintained at the University of California, San Francisco. Mice lacking *Sphk2* and carrying *LoxP* flanked *Sphk1* were on a B6/129 mixed background (Pappu et al., 2007). *Lyve-1 Cre* knockin mice on a B6/129 mixed background was generated as described in Suppl. Fig. 1. Rosa26-YFP reporter mice (Srinivas et al., 2001) were provided by Dr. Nigel Killeen. To generate bone marrow chimeras, recipient mice were lethally irradiated with either 1300 rads in two doses separated by 3 hours and injected with $5x10^6$ wild-type bone marrow cells prepared from CD45.1+ donor. In some experiments, ~$2x10^7$ cells per mL were labeled with 3.3 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen/Molecular Probes) or 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR, Invitrogen/Molecular Probes; (10 μM) in RPMI1640 containing 2% FCS for 20 minutes at 37°C then washed by spinning through a layer of fetal calf

serum. Labeled cells were then resuspended at ~2x10^7 cells per mL and treated with 10 ng/mL of Oligomer B or PTX at 37°C for 10 min, washed twice in warm RPMI, 2% FCS, and 10 mM Hepes, and then transferred to recipient mice. Lymph collection was performed as described (Matloubian et al., 2004). Briefly, under a stereomicroscope, lymph was drawn from the cysterna chyli using a fine borosilicate glass microcapillary pipette (Sutter Instrument). Cell numbers determined by flow cytometry were divided by the volume of collected lymph to determine the concentration. Protocols were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco.

Isolation of lymphatic endothelial cells. Lymph nodes removed from mice were minced into small pieces and added into RPMI 1640 medium containing 2% fetal calf serum (FCS), 10mM HEPES, 100ug/mL DNase, and 0.2 mg/mL Blendzyme 2 (Roche). The samples were then incubated at 37C for 25 minutes, rotating. Midway through the digestion, samples were passed through glass Pasteur pipets multiple times to help break up the pieces of lymph nodes. The digestion was stopped by addition of EDTA and FCS to a final concentration of 10mM and 10%, respectively. The samples were filtered through 100uM cell strainers and centrifuged at 450g for 7 minutes. Cell pellets were resuspended RPMI 1640 medium containing 2% FCS, 10mM HEPES, and 5mM EDTA for analysis.

S1P bioassay. Assay was performed as described by Pappu et. al. 2007. Briefly, platelet-poor plasma or cell-depleted lymph was titrated into RPMI 1640

containing 10mM HEPES and 0.5% fatty-acid free BSA (Calbiochem) in a 96-well U-bottom plate. $4x10^4$ WEHI231 cells stably expressing FLAG-tagged S1P1 (Lo et al., 2005) were added to each well, and the plate was incubated for 40 minutes at 37°C. Cells were analyzed by flow cytometry to measure surface FLAG-S1P1 level, using the M2-FLAG antibody (Sigma). Lymph was drawn as described above into Alsever solution and cells were removed by centrifugation. Platelet-poor plasma was prepared by centrifuging whole blood for 10 minutes at 630g at room temperature.

Immunohistochemical and Flow Cytometric Analysis. Seven μm cryostat sections were fixed and stained as described (Reif et al., 2002). CFSE labeled cells were visualized in sections with alkaline-phosphatase conjugated anti-fluorescein antibodies (Roche). Congenic transferred lymphocytes were visualized by staining with biotinylated antibodies to CD45.1 (clone A20) or CD45.2 (Clone 104). LYVE-1 specific antibody Mab22 was generated as described (Pham et al., 2008) and was from R&D Systems. Anti-murine gp38 hybridoma was from ATCC. For visualization of LYVE-1^+ structures in sections, either unconjugated or biotinylated Mab22 was used. Lymphocyte preparations were stained with various fluorochrome conjugated antibodies purchased from BD Pharmingen or anti-$S1P_1$ as described (Lo et al., 2005) and data were acquired on an FACS LSRII (Beckton Dickinson) and analyzed with FlowJo software (Treestar).

Immunofluorescence analysis. Seven μm sections were prepared from paraformaldehyde-fixed tissues, dried, and blocked with "immunomix" (1X PBS, 5% normal serum, 0.3% Triton-X100, 0.2% BSA, and 0.1% sodium azide) for at least 1 hour. Sections were stained with primary antibodies in "immunomix": biotinylated anti-LYVE-1 (R&D Systems) and rabbit anti-GFP (Invitrogen) for 3 hours to overnight. After 2 washes with 1X PBS, sections were then stained with Cy3 and Cy5 conjugated antibodies (Jackson Immunoresearch) and DAPI for 3 hours. Sections were analyzed on an Axiovert Z1 miscroscope (Zeiss). For whole mount staining, mice were perfused for 2 min with fixative (1% paraformaldehyde in PBS, pH 7.4) from a canula inserted through the left ventricle into the aorta. The tracheas and diaphragms were removed and immersed in fixative for 1 hour at 4C. Tissues were washed and stained as described previously (Baluk et al., 2007).

References:

Azzali, G. (2003). Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. Immunological reviews *195*, 178-189.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E., and McDonald, D.M. (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med *204*, 2349-2362.

Baluk, P., Tammela, T., Ator, E., Lyubynska, N., Achen, M.G., Hicklin, D.J., Jeltsch, M., Petrova, T.V., Pytowski, B., Stacker, S.A.*, et al.* (2005). Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. The Journal of clinical investigation *115*, 247-257.

Belisle, C., and Sainte-Marie, G. (1981). Tridimensional study of the deep cortex of the rat lymph node. III. Morphology of the deep cortex units. Anat Rec *199*, 213-226.

Camerer, E., Regard J.B., Cornilissen I., Srinivasan Y., Duong D.N., Palmer D., Pham, T.H., Pappu R., Coughlin S.R. (2009). Sphingosine-1-phosphate in the plasma compartment regulates basal and inflmmation-induced vascular leak in mice. The Journal of clinical investigation.

Compton, C.C., and Raviola, E. (1985). Structure of the sinus-lining cells in the popliteal lymph node of the rabbit. Anat Rec *212*, 408-423.

Gale, N.W., Thurston, G., Hackett, S.F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M.H., Jackson, D.*, et al.* (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Developmental cell *3*, 411-423.

Gordon, E.J., Gale, N.W., and Harvey, N.L. (2008). Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE-1 is also expressed on embryonic blood vessels. Dev Dyn *237*, 1901-1909.

Grigorova, I.L., Schwab, S.R., Phan, T.G., Pham, T.H., Okada, T., and Cyster, J.G. (2009). Cortical sinus probing, S1P1-dependent entry and flow-based capture of egressing T cells. Nat Immunol *10*, 58-65.

He, Y. (1985). Scanning electron microscope studies of the rat mesenteric lymph node with special reference to high-endothelial venules and hitherto unknown lymphatic labyrinth. Arch Histol Jpn *48*, 1-15.

Ito, K., Anada, Y., Tani, M., Ikeda, M., Sano, T., Kihara, A., and Igarashi, Y. (2007). Lack of sphingosine 1-phosphate-degrading enzymes in erythrocytes. Biochemical and biophysical research communications *357*, 212-217.

Jackson, D.G. (2004). Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. Apmis *112*, 526-538.

Jakubzick, C., Bogunovic, M., Bonito, A.J., Kuan, E.L., Merad, M., and Randolph, G.J. (2008). Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. J Exp Med *205*, 2839-2850.

Jang, C., Koh, Y.J., Lim, N.K., Kang, H.J., Kim, D.H., Park, S.K., Lee, G.M., Jeon, C.J., and Koh, G.Y. (2009). Angiopoietin-2 exocytosis is stimulated by sphingosine-1-phosphate in human blood and lymphatic endothelial cells. Arteriosclerosis, thrombosis, and vascular biology *29*, 401-407.

Karpanen, T., and Alitalo, K. (2008). Molecular biology and pathology of lymphangiogenesis. Annual review of pathology *3*, 367-397.

Kawahara, A., Nishi, T., Hisano, Y., Fukui, H., Yamaguchi, A., and Mochizuki, N. (2009). The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. Science *323*, 524-527.

Kennedy, S., Kane, K.A., Pyne, N.J., and Pyne, S. (2009). Targeting sphingosine-1-phosphate signalling for cardioprotection. Current opinion in pharmacology *9*, 194-201.

Kono, M., Allende, M.L., and Proia, R.L. (2008). Sphingosine-1-phosphate regulation of mammalian development. Biochimica et biophysica acta *1781*, 435-441.

Le Stunff, H., Milstien, S., and Spiegel, S. (2004). Generation and metabolism of bioactive sphingosine-1-phosphate. Journal of cellular biochemistry *92*, 882-899.

Lee, J.F., Zeng, Q., Ozaki, H., Wang, L., Hand, A.R., Hla, T., Wang, E., and Lee, M.J. (2006). Dual roles of tight junction-associated protein, zonula occludens-1, in sphingosine 1-phosphate-mediated endothelial chemotaxis and barrier integrity. J Biol Chem *281*, 29190-29200.

Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell *99*, 301-312.

Link, A., Vogt, T.K., Favre, S., Britschgi, M.R., Acha-Orbea, H., Hinz, B., Cyster, J.G., and Luther, S.A. (2007). Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. Nat Immunol *8*, 1255-1265.

Lo, C.G., Xu, Y., Proia, R.L., and Cyster, J.G. (2005). Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J Exp Med *201*, 291-301.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G.F., Spiegel, S., and Proia, R.L. (2005). Essential role for sphingosine kinases in neural and vascular development. Molecular and cellular biology *25*, 11113-11121.

Oliver, G., and Srinivasan, R.S. (2008). Lymphatic vasculature development: current concepts. Annals of the New York Academy of Sciences *1131*, 75-81.

Osborne, N., Brand-Arzamendi, K., Ober, E.A., Jin, S.W., Verkade, H., Holtzman, N.G., Yelon, D., and Stainier, D.Y. (2008). The spinster homolog, two of hearts, is required for sphingosine 1-phosphate signaling in zebrafish. Curr Biol *18*, 1882-1888.

Paik, J.H., Skoura, A., Chae, S.S., Cowan, A.E., Han, D.K., Proia, R.L., and Hla, T. (2004). Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. Genes & development *18*, 2392-2403.

Pappu, R., Schwab, S.R., Cornelissen, I., Pereira, J.P., Regard, J.B., Xu, Y., Camerer, E., Zheng, Y.W., Huang, Y., Cyster, J.G., and Coughlin, S.R. (2007). Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science *316*, 295-298.

Pham, T.H., Okada, T., Matloubian, M., Lo, C.G., and Cyster, J.G. (2008). S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. Immunity *28*, 122-133.

Podgrabinska, S., Braun, P., Velasco, P., Kloos, B., Pepper, M.S., and Skobe, M. (2002). Molecular characterization of lymphatic endothelial cells. Proceedings of the National Academy of Sciences of the United States of America *99*, 16069-16074.

Reif, K., Ekland, E.H., Ohl, L., Nakano, H., Lipp, M., Forster, R., and Cyster, J.G. (2002). Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. Nature *416*, 94-99.
Rosen, H., and Goetzl, E.J. (2005). Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nature reviews *5*, 560-570.

Sanchez, T., Estrada-Hernandez, T., Paik, J.H., Wu, M.T., Venkataraman, K., Brinkmann, V., Claffey, K., and Hla, T. (2003). Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. J Biol Chem *278*, 47281-47290.

Sanna, M.G., Wang, S.K., Gonzalez-Cabrera, P.J., Don, A., Marsolais, D., Matheu, M.P., Wei, S.H., Parker, I., Jo, E., Cheng, W.C.*, et al.* (2006).

Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P(1) antagonist in vivo. Nat Chem Biol *2*, 434-441.

Schwab, S.R., and Cyster, J.G. (2007). Finding a way out: lymphocyte egress from lymphoid organs. Nat Immunol *8*, 1295-1301.

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J.G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science *309*, 1735-1739.

Sinha, R.K., Park, C., Hwang, I.Y., Davis, M.D., and Kehrl, J.H. (2009). B lymphocytes exit lymph nodes through cortical lymphatic sinusoids by a mechanism independent of sphingosine-1-phosphate-mediated chemotaxis. Immunity *30*, 434-446.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC developmental biology *1*, 4.

Venkataraman, K., Lee, Y.M., Michaud, J., Thangada, S., Ai, Y., Bonkovsky, H.L., Parikh, N.S., Habrukowich, C., and Hla, T. (2008). Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circulation research *102*, 669-676.

Yoon, C.M., Hong, B.S., Moon, H.G., Lim, S., Suh, P.G., Kim, Y.K., Chae, C.B., and Gho, Y.S. (2008). Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. Blood *112*, 1129-1138.

Acknowledgments

We thank Jinping An for excellence care of mouse colonies and Tal Arnon for discussion. T.H.M.P. is supported by the Boyer Program in the Biochemical Sciences and the UCSF Medical Scientist Training Program. J.G.C. is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by grants from the NIH. The authors declare they have no financial conflicts of interest.

Figure Legends

Figure 1. Ablation of lymph S1P by conditional deletion of *Sphk1* in *Sphk2*-deficient mice. (A) Flow cytometric analysis showing S1P1 on $CD4^+$ $CD62L^{hi}$ T cells from indicated circulatory fluids and tissues. Δ indicates *Lyve-1 Cre*+ *Sphk1*$^{f/-\text{or } f}$ *Sphk2*$^{-/-}$ mice; C indicates littermate control. Lymph node (LN), lymph (Lym), spleen (Spl), and blood (Bld). Shaded histograms show staining with control antibody of cells from (LN) and (Bld). (B) Measurement of S1P level by bioassay. Lymph fluid and plasma samples were prepared (see Methods) and titrated onto WEHI231 cells expressing FLAG-S1P1. The x-axis shows dilution of the samples. The y-axis indicates mean fluorescence intensity (MFI) of staining against anti-FLAG antibody from flow cytometric analysis. Data are representative of at least three experiments.

Figure 2. Efficiency of *Lyve-1* CRE-mediated gene deletion. (A) Isolation and identification of lymph node LECs by flow cytometry. CD31 and gp38 expression allows separation of LECs from other $CD45^-$ cells: T-zone reticular cells (TRCs), blood endothelial cells (BECs), and double-negative stromal cells (others). Histogram plots show level of LYVE-1 on indicated cell populations. Control refers to an isotype control for the anti-LYVE-1 antibody. (B) Flow cytometric analysis of YFP expression in cells isolated from lymph nodes of *LoxP*-STOP-*LoxP* Rosa26YFP reporter mice carrying the *Lyve-1 Cre* knock-in gene. The four cells populations are gated following the scheme in A. Peripheral lymph nodes (pLN) include axillary, brachial, and inguinal nodes; mesenteric lymph nodes (mLN). Shaded histogram shows background YFP signal from Cre-negative

control cells. (C) Expression of S1P metabolic genes in lymphatic endothelial cells (LECs). LECs, TRCs, and BECs were sorted from lymph node samples using the scheme in A. Quantitative RT-PCR analysis was performed for the indicated genes on the sorted cells, along with splenic tissue (Spl). Asterisks indicate undetectable signals. Data are representative from three separate sorts, with each gene expression measured at least twice. (D) Immunofluorescence analysis of YFP expression in lymph node tissue of *Lyve-1 Cre*+ Rosa26YFP mice. Cre+ and Cre- control mice were lethally irradiated and reconstitute with wild-type CD45.1+ bone marrow. Seven μM sections were prepared from paraformaldehyde fixed lymph nodes and stained for LYVE-1 (red) and YFP (green). DAPI (blue) visualizes cell nuclei. Objective magnification, 40X. Data are representative of three experiments.

Figure 3. Lack of contribution of myeloid cells to lymph S1P. (A & B) Expression of LYVE-1 by lymph node macrophages. (A) Flow cytometric analysis of enzyme digested lymph node cells to detect CD11b and CD11c expressing sub-populations. In the plot on the right, populations defined by gates are numbered 1 through 6. (B) Staining of LYVE-1 on the six cell populations shown in (A). Red histogram, staining with isotype control antibody; blue histogram, staining with anti-LYVE-1 antibody. (C) Degree of chimerism of myeloid cells at high dose of lethal irradiation. Lethally irradiated $CD45.2^+$ *Lyve-1 Cre*+ *Sphk*-deficient mice and controls were reconstituted with wild-type CD45.1+ bone marrow. Flow cytometric analysis showing the fraction of donor-derived cells in each of the six populations indicated in (A). Numbers refer to the percent cells in

the indicated gates. (D) Flow cytometric analysis showing S1P1 on $CD4^+$ $CD62L^{hi}$ T cells from the lymph and lymph node of the chimeric mice in (C). *Sphk* Δ indicates *Lyve-1* Cre+ Sphk-deficient host; C indicates control host. Lymph node (LN); lymph (Lym). Shaded histogram shows staining of lymph node cells from an FTY720 treated control mouse. (E) Measurement of lymph S1P by bioassay. Lymph and plasma samples were titrated onto FLAG-S1P1 WEHI231 cells and staining for FLAG as in Figure 1B. Data are representative of two experiments (A & B); and three experiments (C-E).

Figure 4. Impaired lymphocyte egress in *Lyve-1 Cre Sphk*-deficient mice. (A-E) Cells numbers in indicated fluids and tissues in *Lyve-1 Cre*+ *Sphk*-deficient and control mice. Lymph node count was from a pool of two axillary, brachial, and inguinal nodes. Enumerated CD4+, CD8+, and CD19+ were $CD62L^{hi}$. Points indicate data from individual mice; white (control mice) and black (Sphk-deficient mice) bars represent means. (F-G) Empty LYVE-1+ sinuses in *Lyve-1 Cre*+ *Sphk*-deficient mice. Seven μM sections prepared from lymph node (F) and Peyer's patch (G) were stained for LYVE-1 (brown) and CD3 (blue) or B220 (blue). *Sphk* Δ indicates *Lyve-1 Cre*+ *Sphk*-deficient mice; Control indicates littermate control mice. Objective magnification, 10X. Data are representative of at least three experiments.

Figure 5. PTX treatment facilitates lymphocyte egress and localization in cortical sinuses in *Lyve-1 Cre+ Sphk*-deficient mice. (A-C) Splenocytes were treated with either pertussis toxin (PTX) or Oligomer-B (OB) as control and co-transferred into recipient hosts. Twenty-two hours later, transferred cell numbers

were determined in the lymph and lymph nodes of *Lyve-1* Cre+ Sphk-deficient (*Sphk* Δ) and control hosts. (A) Flow cytometric analysis of transferred T and B cells present in the lymph. Numbers refer to the percent of cells in the indicated gates. (B) Frequency of transferred PTX and OB treated cells in peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), and the lymph (Lym). (C) The total numbers of transferred CD4+ and CD8+ cells in the lymph from the same experiments as A & B are plotted. (B-C) Points indicate data from individual mice; bars indicate means. (D) Presence of PTX and OB treated cells in cortical sinuses. Purified T cells were treated and co-transferred into recipient hosts as in A-C. Sections stained for LYVE-1 (brown) and transferred cells with indicated treatment (blue). Transferred cells located inside sinuses are marked by arrows. Objective magnification, 20X. Images are representative from three experiments.

Figure 6. Effect of lymphatic *Sphk*-deficiency on lymphatic vasculature. (A & B) Confocal images showing lymphatic vessels stained with antibody against LYVE-1 in whole mount of mouse trachea. White squares in (A) indicate the regions enlarged in (B). Arrowheads point to the jagged appearance of the lymphatic vessels in *Lyve-1 Cre*+ *Sphk*-deficient mice. (C & D) Confocal images showing button-like pattern of VE-cadherin at endothelial cell-cell junctions of the diaphragm initial lymphatics. White squares in (C) indicate the regions enlarged in (D). Arrowheads mark VE-cadherin+ “buttons” in the control and a corresponding junctional region in the *Lyve-1 Cre*+ *Sphk*-deficient. Objective magnification: 10X (A), 40X (B, C & D). Data are representative of six experiments (n=6 mice).

Supplementary Figures

Supplemental Figure 1. Map of *Lyve-1 EGFP-hCRE* construct. (A) A 5.4kb and a 3.4kb fragment homologous to the 5' and the 3' end, respectively, of the *Lyve-1* 3' UTR was PCR-amplified and subcloned into pIRES-GFP-hCre-FNF vector using BD In-Fusion Dry-Down PCR cloning kit. The resulting vector construct was linearized by SacII and electroporated into E14 (129/Ola) ES cells. Clones were screened by 3' end long PCR using Roche Long Template PCR system. (B) Homologous recombination was confirmed by Southern blot with 5' and 3' probes, the targeted allele giving rise to a 14 kb Bam H1 fragment with the 5' probe and a 9.6 kb HindIII fragment with the 3' probe. Three of the successfully targeted clones were injected into C57BL/6 blastocysts. Chimeras were bred to B6 mice and germline transmission was confirmed by allele-specific PCR using the following primers: Cre-SFnew1 (*GAACTACATCAGAAACCTGGA*) and LYVE-1-KI-QR (*AGAGGGCTCCTTCTGGTGA*). The Neomycin resistance cassette was removed by intercrossing with mice expressing Flip recombinase in the germline.

Supplemental Figure 2. (A & B) Efficiency of *Lyve-1 Cre*-mediated gene deletion. Immunofluorescence analysis of YFP and LYVE-1 expression in ear dermis (A) and small intestine (B) of *Lyve-1 Cre* Rosa26YFP reporter mice that had been lethally irradiated and reconstituted with YFP- bone marrow. (C) Promotion of lymphocyte egress into the lymph is not dependent on a hematopoietic source of S1P. Lethally irradiated *Lyve-1 Cre Sphk*-deficient and

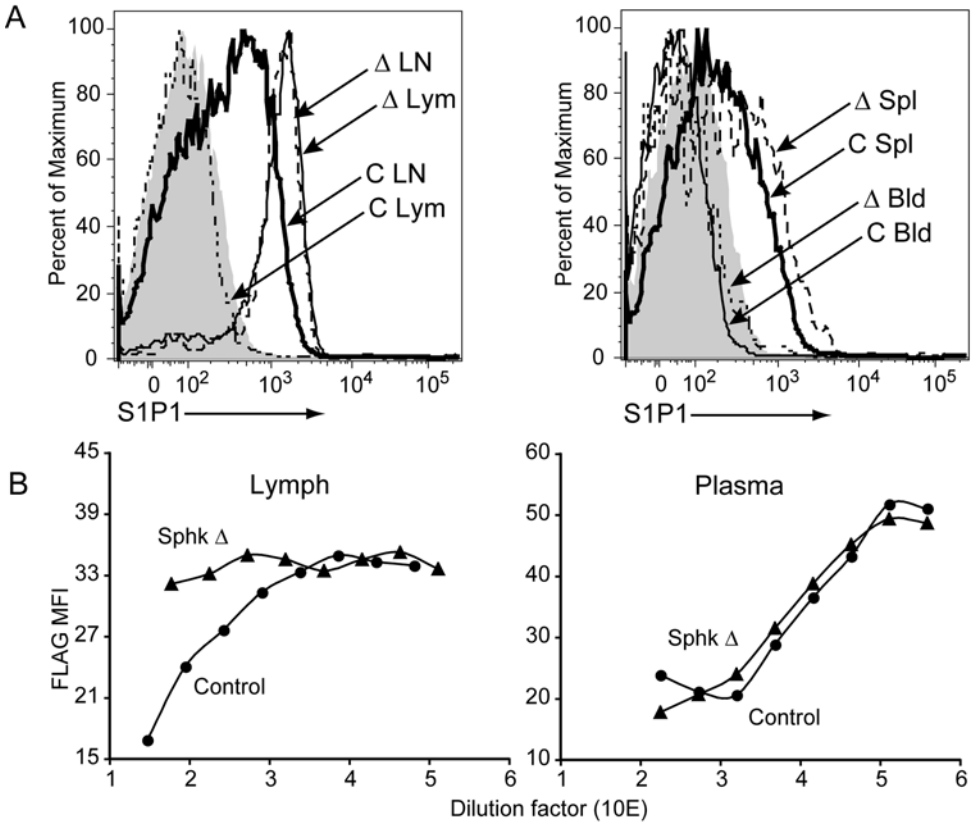
control mice were reconstituted with wild-type bone marrow. The cell numbers in the lymph of these chimeric mice were determined. Points indicate data from individual mice; white (control mice) and black (*Sphk*-deficient mice) bars represent means. (D) Differential effect of lymphatic *Sphk* deletion on local tissue S1P availability. Flow cytometric analysis showing S1P1 on $CD4^+$ $CD62L^{hi}$ T cells from the indicated tissues. *Sphk* Δ indicates *Lyve-1 Cre Sphk*-deficient mice; Control indicates littermate control. Shaded histograms show staining with control antibody. Objective magnification (A & B): 40X.

Supplemental Figure 3. Cortical sinus emptying following FTY720 treatment. Sections of peripheral lymph nodes from mice treated 6 hours earlier with carrier (PBS) or FTY720 (1mg/kg) showing immunolabeling of LYVE-1 (green) and CD4 (red) and staining of nuclei with DAPI (blue). Objective magnification, 20X.

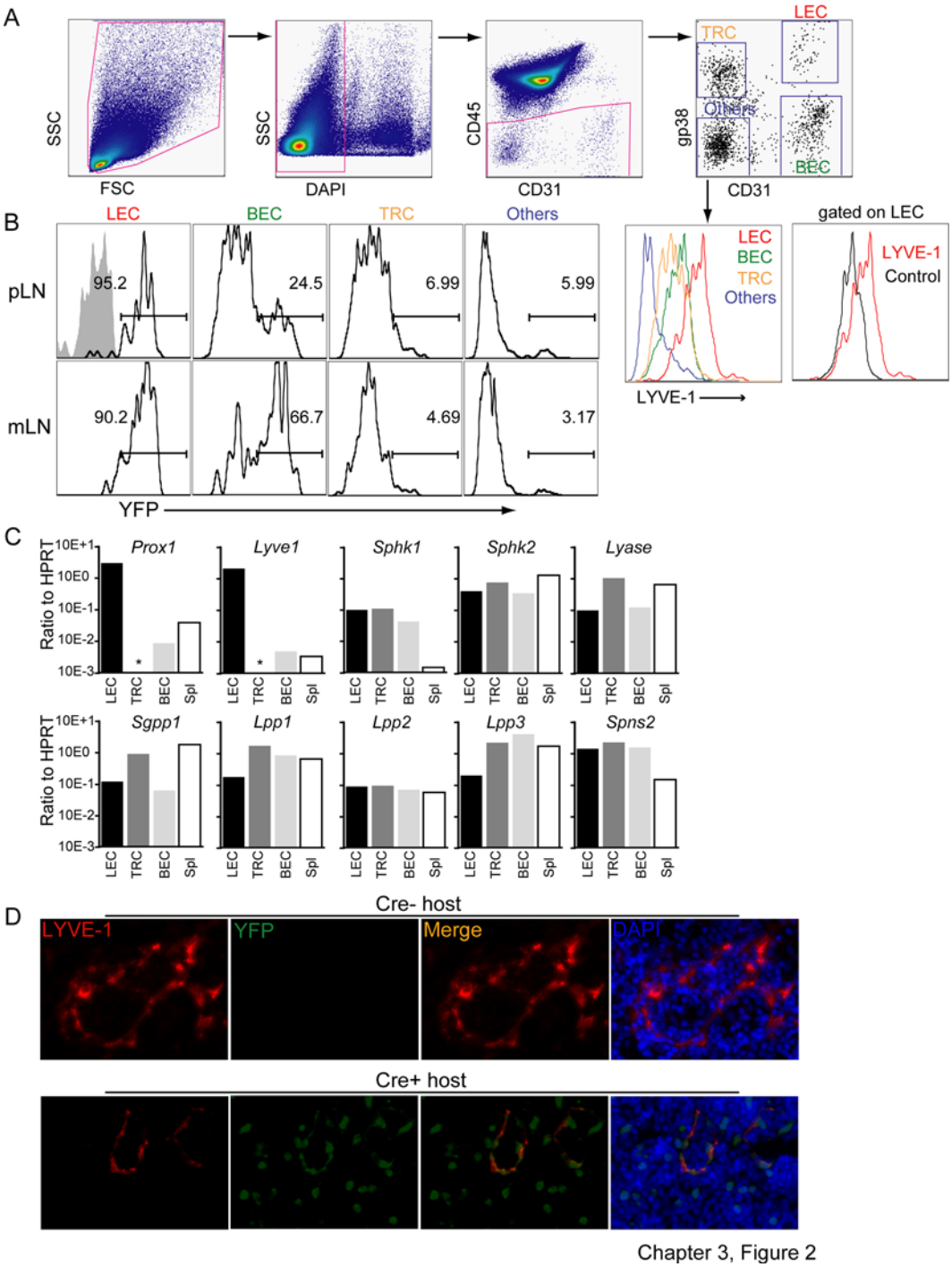
Supplemental Figure 4. Recovery of B cell egress in *Lyve-1 Cre Sphk*-deficient mice by PTX treatment and further examples of cell distribution in lymph node cortical sinusoids. (A & B) Splenocytes were treated with either pertussis toxin (PTX) or Oligomer-B (OB) as control and co-transferred into recipient hosts. Twenty-two hours later, transferred cell numbers were determined in the lymph and lymph nodes of *Lyve-1 Cre Sphk*-deficient (Δ) and control (C) hosts. (A) Frequency of transferred PTX and OB treated B cells in peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), and the lymph (Lym). (B) The total numbers of transferred CD19+ cells in the lymph from the same experiments as (A) are plotted. Points indicate data from individual mice;

bars represent means. (C) Further examples of the cortical sinus analysis shown in Fig. 6D. Purified T cells were treated with PTX or Oligomer-B and co-transferred into lymphatic Sphk-deficient or control hosts. Sections of lymph nodes were stained for LYVE-1 (brown) and transferred cells with the indicated treatment (blue). Objective magnification, 20X.

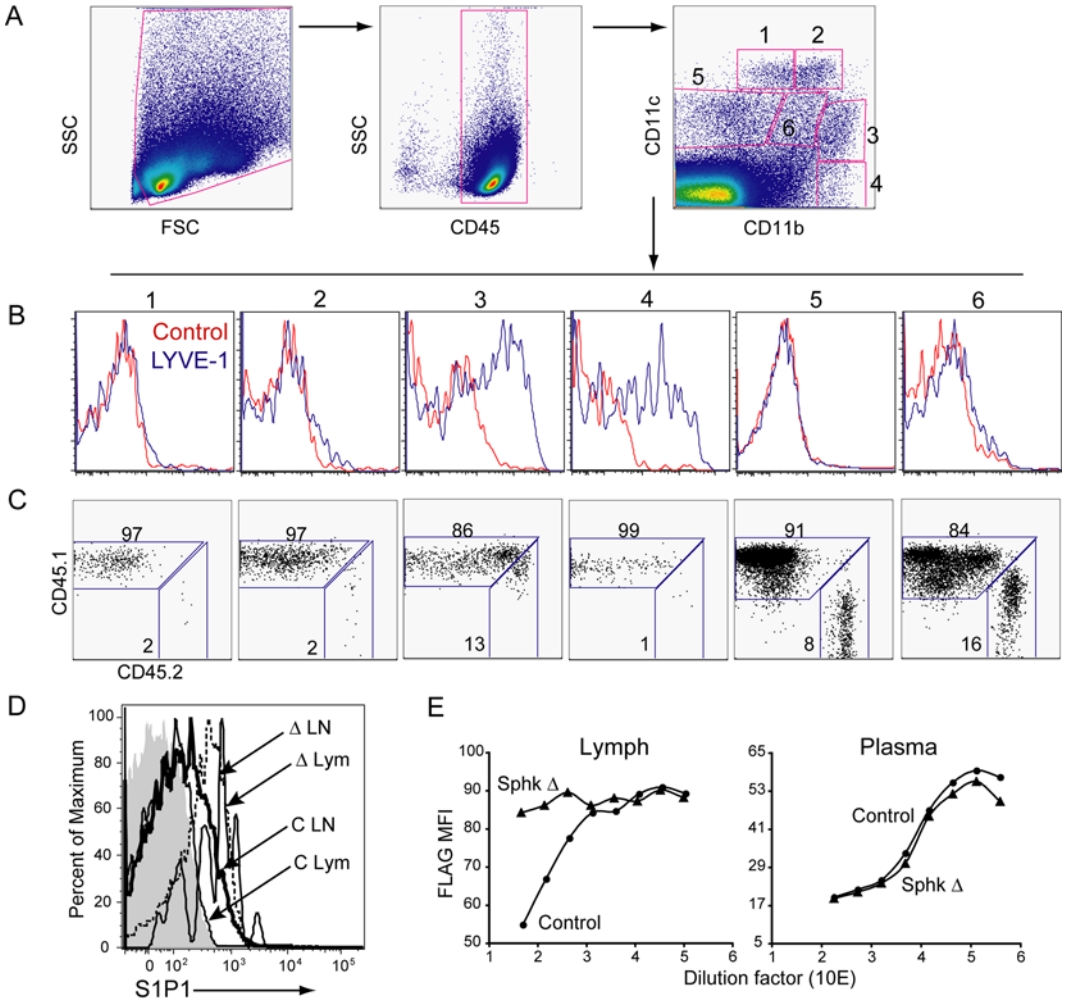
Supplemental Figure 5. More examples of the effects of *Sphk* -deficiency on lymphatic vasculature. (A & B) Confocal images showing lymphatic vessels stained with antibody against LYVE-1 in whole mount of mouse trachea. White squares in (A) indicate the regions enlarged in (B). (C-E) Confocal images showing button-like pattern of VE-cadherin at endothelial cell-cell junctions of the diaphragm initial lymphatics (C, D) and LYVE-1 staining in diaphragm initial lymphatics (E). White squares in (C) indicate the regions enlarged in (D). (F) Expression of S1P receptors in LECs. LECs, TRCs (T-zone reticular cells), and BECs (blood endothelial cells) were sorted from lymph node samples using the same surface markers as in Figure 2A. Quantitative RT-PCR analysis for indicated genes on the sorted cells, along purified B cells. Asterisks indicate undetectable signals. Analysis of *S1p5* expression showed undetectable signals in most samples. Data are representative of two experiments. Objective magnification: 10X (A & E), 40X (B, C & D). Data are representative of six experiments (n=6 mice).



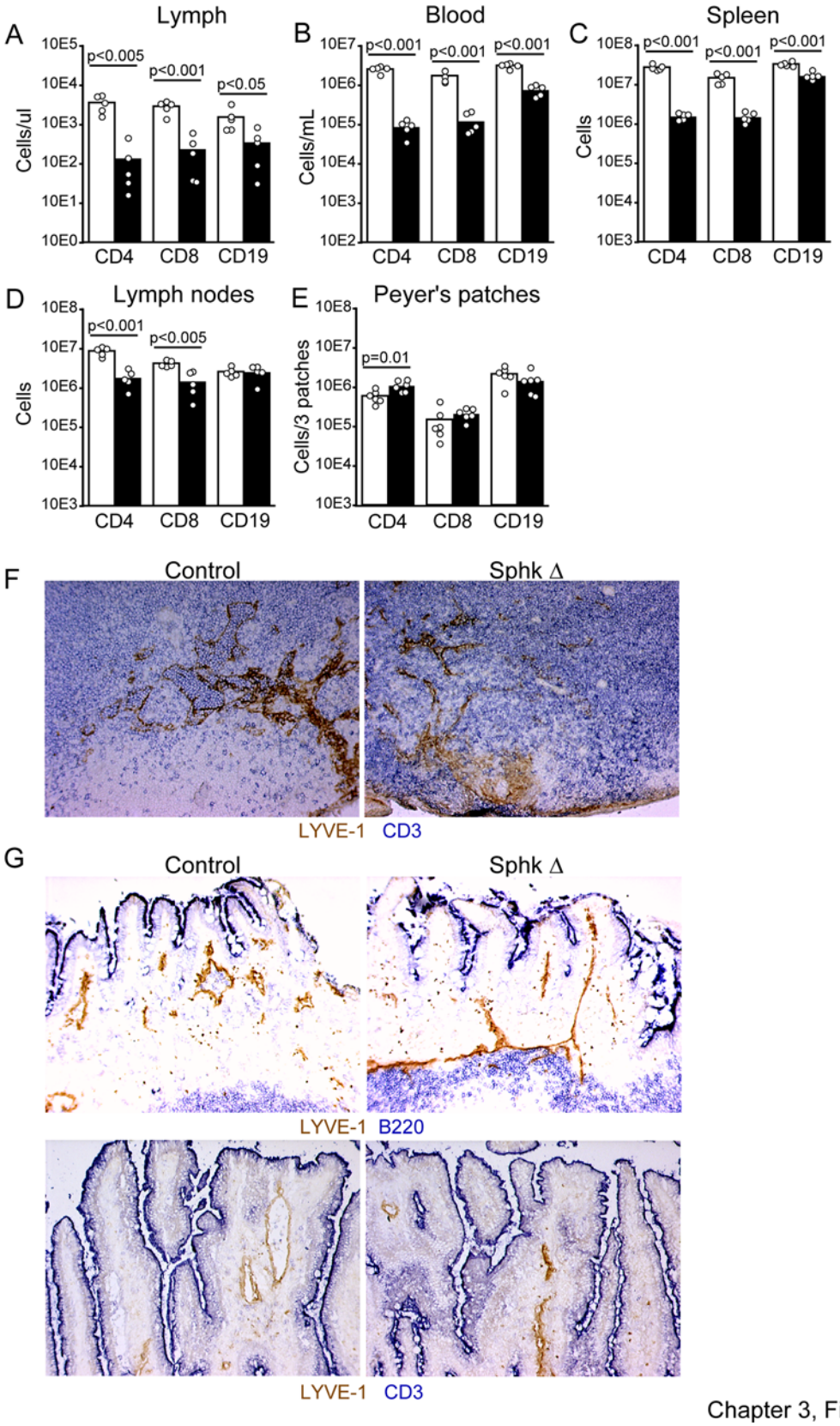
Chapter 3, Figure 1



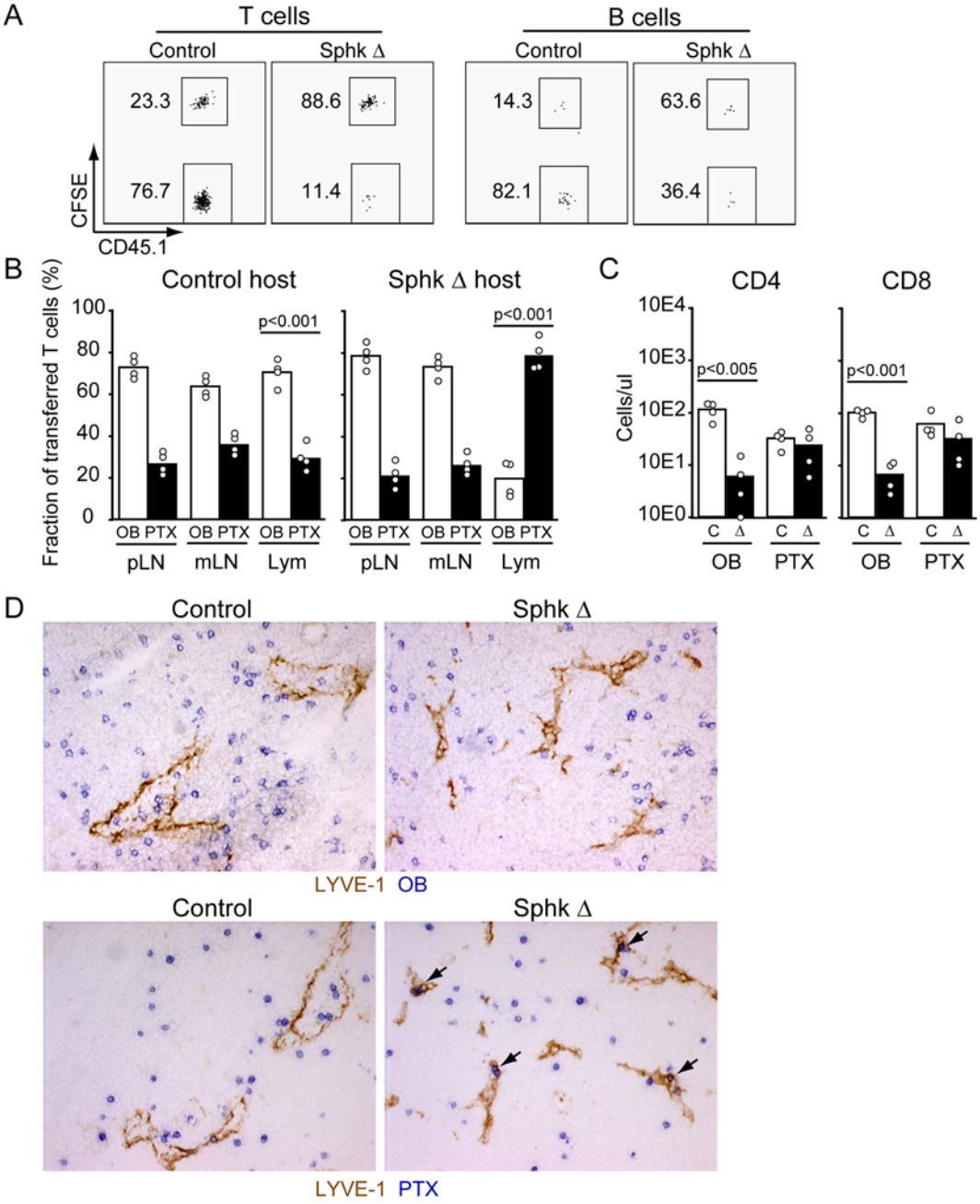
Chapter 3, Figure 2



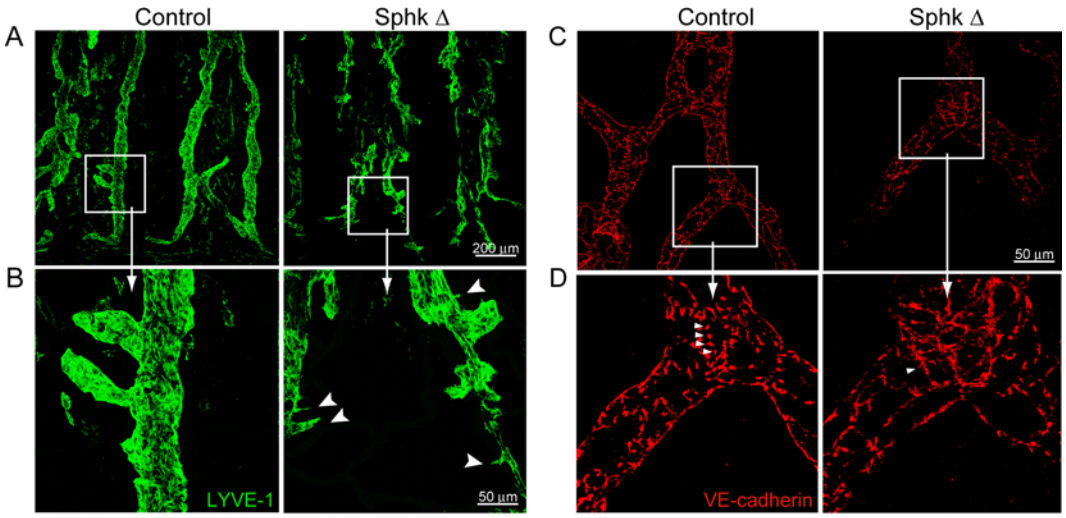
Chapter 3, Figure 3



Chapter 3, Figure 4

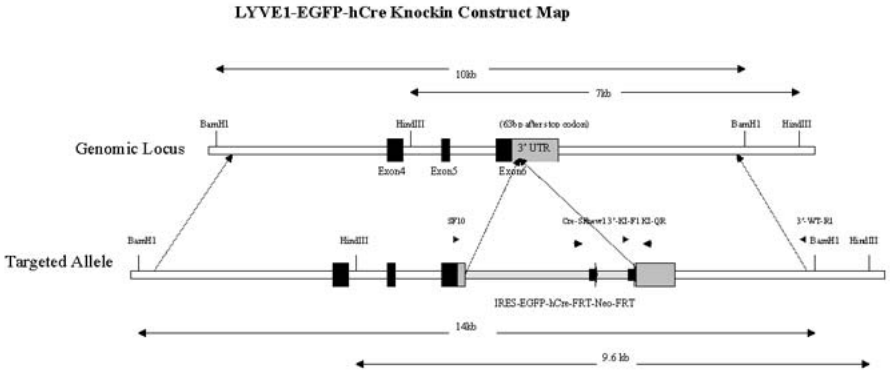


Chapter 3, Figure 5

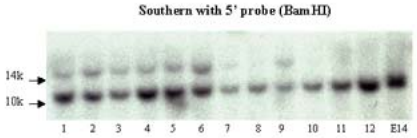


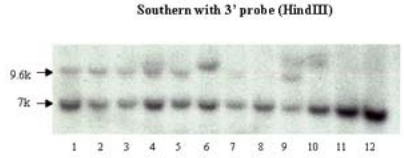
Chapter 3, Figure 6

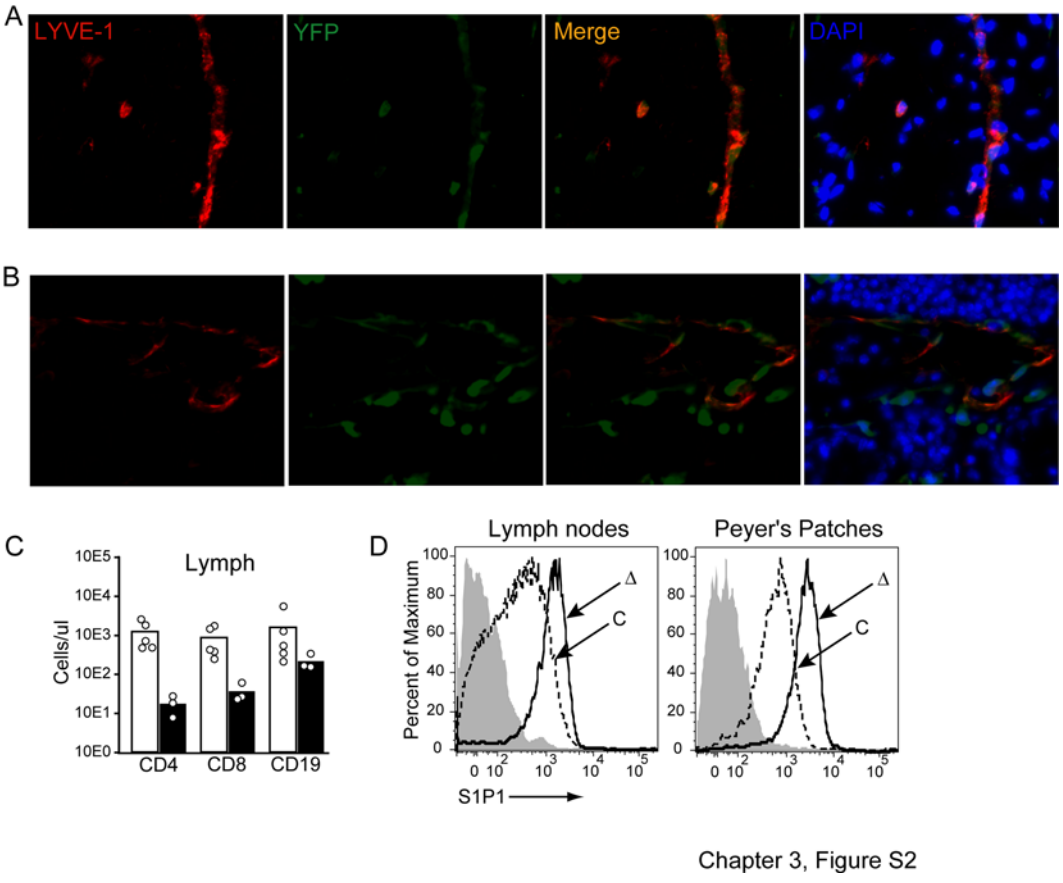
A.


LYVE1-EGFP-hCre Knockin Construct Map
10kb
7kb
BamH1
HindIII
(63bp after stop codon)
BamH1
HindIII
Genomic Locus
3' UTR
Exon4
Exon5
Exon6
SP10
BamH1
HindIII
3'-WT-R1
BamH1
HindIII
Targeted Allele
IRES-EGFP-hCre-FRT-Neo-FRT
14kb
9.6 kb

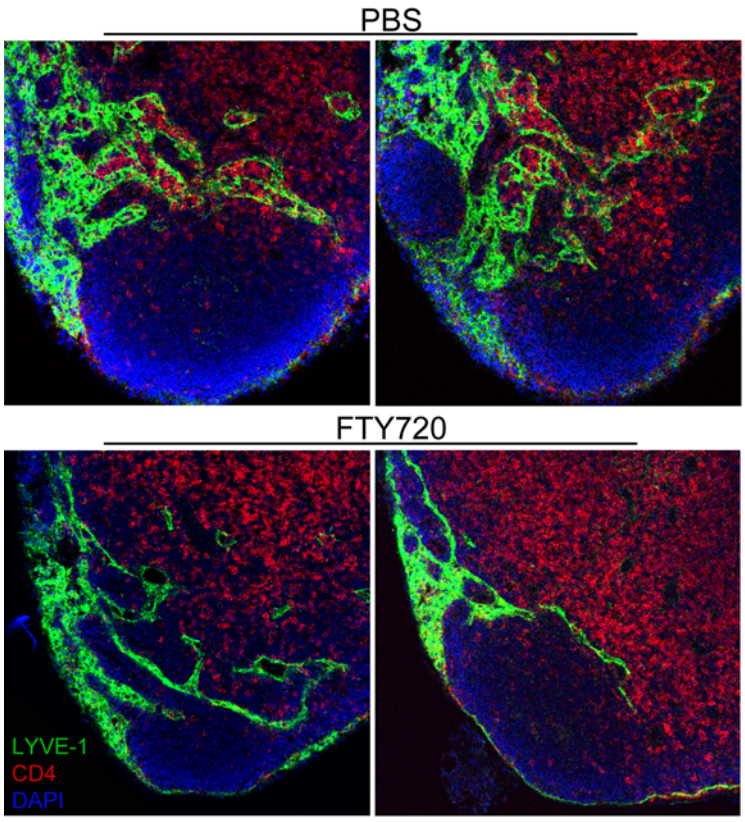
B.


Southern with 5' probe (BamHI)
14k
10k
1 2 3 4 5 6 7 8 9 10 11 12 E14

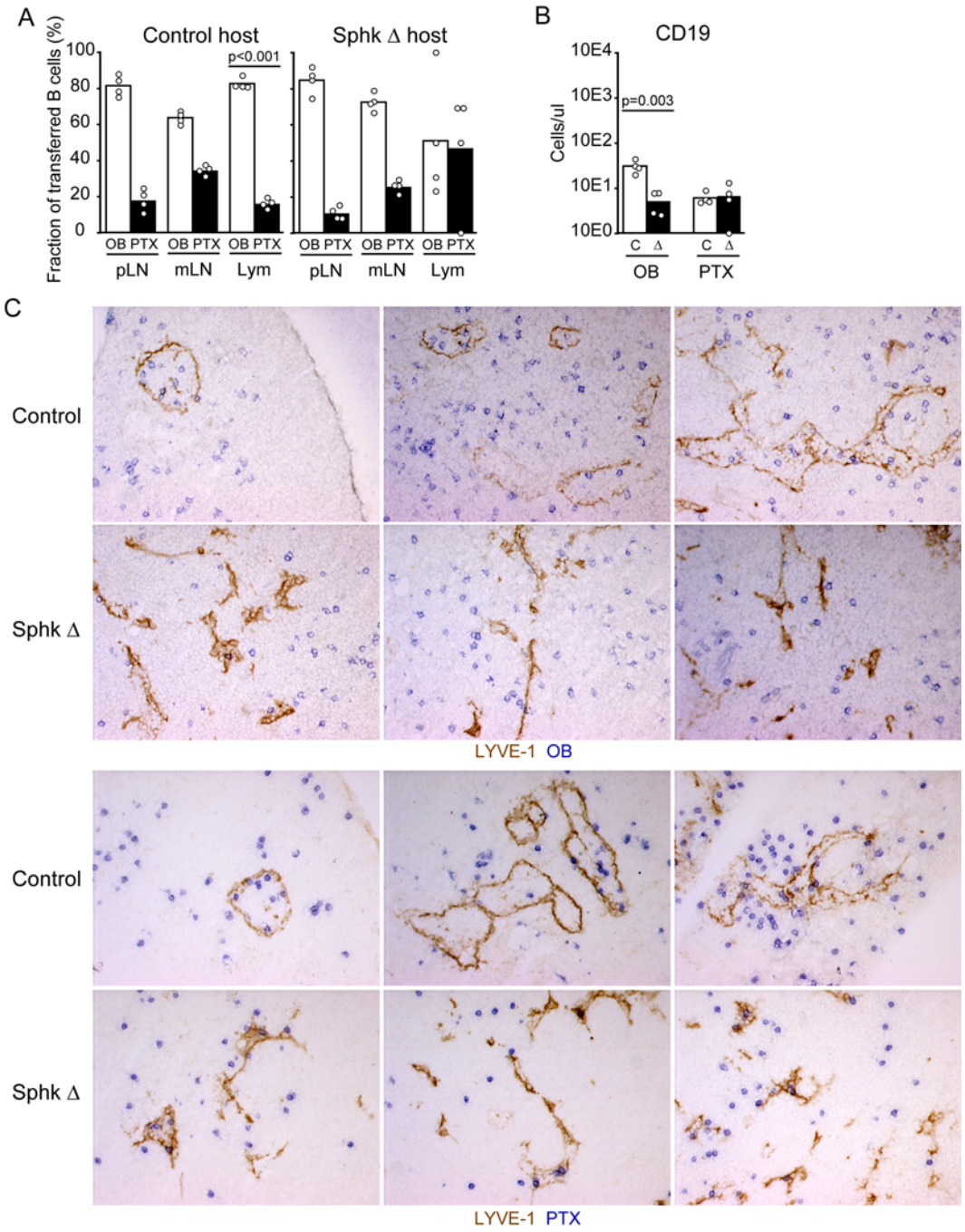

Southern with 3' probe (HindIII)
9.6k
7k
1 2 3 4 5 6 7 8 9 10 11 12



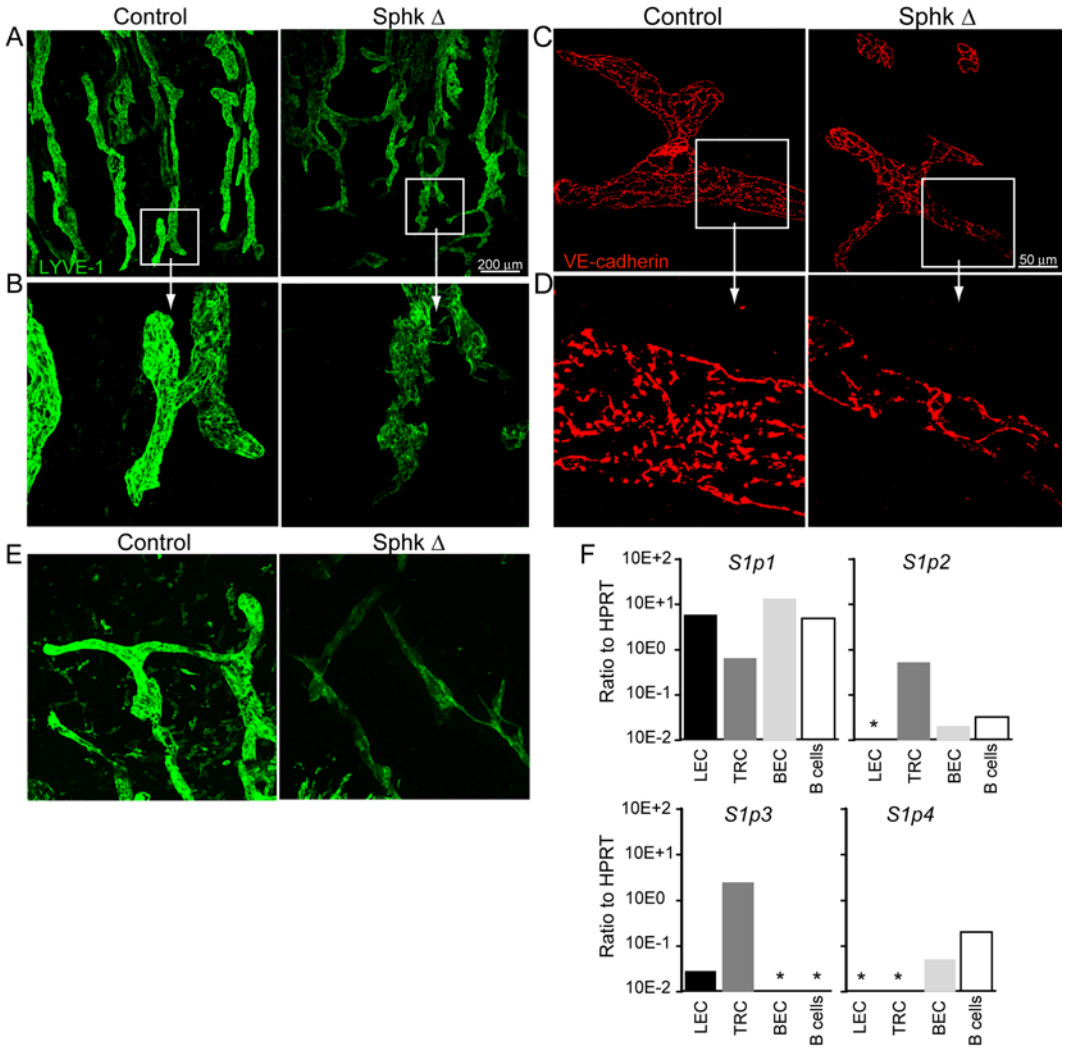
Chapter 3, Figure S2



Chapter 3, Figure S3



Chapter 3, Figure S4



Chapter 3, Figure S5

CHAPTER 4

Conclusion

Lymphocyte egress is essential for normal immune functions and a target of pharmacological agents being developed for immunosupression therapy. In the studies presented, we have defined a major mechanism by which lymphocyte S1P1 functions to promote lymphocyte egress. Our studies also report a novel function of CCR7 as a lymphocyte retention factor and identify cortical sinuses as anatomical sites at which lymphocyte egress from the lymph nodes occurs. We have defined lymphatic endothelial cells (LECs) as the major cellular source of lymph S1P required for egress from the lymph nodes and provide mechanistic insight into how lymph S1P induces lymphocyte egress. Our findings also help resolve the debate on whether S1P signaling regulates lymphocyte egress principally through the lymphocytes or stromal cells. Finally, we provide the first evidence for a role of S1P signaling in the physiological development of the lymphatic vasculature.

Based on the observations presented here and those from recent published *in vivo* imaging studies (Grigorova et al., 2009), we propose the following model for the early steps of lymphocyte egress from the lymph nodes (Chapter 4, Fig. 1): T cells migrate upon a CCL21+ stromal network composed of fibroblastic reticular cells (FRC) in the lymph node in a “random walk” fashion. Upon encountering a LYVE+ cortical or medullary sinus composed of lymph S1P-producing LECs, T cells simultaneously experiencing CCL21 and S1P gradients extend and retract multiple pseudopods to probe the sinus lumen and intersecting stromal fibers. If the CCR7 retention signal is stronger, a committed pseudopod is projected away from the LYVE-1+ sinus and cells migrate back into

the lymph node parenchyma. This outcome may be favored with T cells that have just entered the lymph node, have reduced S1P1 function due to activation or genetic deficiency, or have more CCR7 due to transgenesis. Alternatively, if the S1P1 egress-promoting signal is stronger, a committed pseudopod is extended in the direction of the sinus and cells transmigrate into this compartment. This outcome may be favored in cells that have fully up-regulated S1P1 after being in the lymph nodes for several hours, or that have reduced CCR7 due to genetic deficiency, desensitization or activation-induced down-modulation. Once inside the sinuses, cells can be captured by flow and carried to the medullary sinuses, and then into the efferent lymph out of the lymph nodes. For simplicity, we represent retention signal with only CCR7 here. Our data suggest that there are additional Gαi-coupled receptors functioning as retention receptors similar to CCR7 that remain to be identified.

Lymphocyte egress from the lymph nodes is a dynamic, multi-step process that includes: 1)cell migration away from T and B zones toward exit structures; 2)transmigration of cells across the egress barrier; and 3) subsequent retention of cells in the lymphatics for transport out of lymph nodes. Lymphocytes going through this series of events during egress also need to navigate through an environment rich in chemoattractants that can misdirect exiting cells away from their target destination inside the lymphatic sinuses. Thus, to mediate efficient egress, lymphocyte S1P1 signaling may operate through one or more mechanisms during the multi-step process: overcoming retention signals that retain cells in the lymphoid tissue parenchyma, promoting

cell migration toward exit structures or transmigration into lymphatic sinuses, or retaining cells inside the lymphatic sinuses. Our data show that removal of Gαi-mediated retention signals by PTX-treatment normalizes loss of S1P1 function and restores egress competence in FTY720-experienced and S1P1-/- T cells, suggesting that the mechanism by which lymphocyte S1P1 promotes egress is to override retention mediated by Gαi-coupled receptors (Pham et al., 2008). Considering that PTX-treatment reduces T cell motility by about 40%-60%, overcoming retention appears to be the major function of lymphocyte S1P1 in promoting egress, as PTX-treated T cells that are S1P1-deficient or have been exposed to FTY720 can achieve between 30%-50% egress efficiency of wild-type, control-treated T cells. Our immunohistochemical analyses demonstrate that lymphocyte S1P1 is required for T cell accumulation in the LYVE-1+ cortical sinuses, suggesting that S1P1 promotes entry of cells into the lymphatics and/or retention within this compartment (Pham et al., 2008). *In vivo* imaging studies have confirmed the requirement of S1P1 for T-cell entry into LYVE-1+ cortical and medullary sinuses in the lymph nodes (Grigorova et al., 2009). Furthermore, the dependence of lymphocyte egress from lymph nodes on a gradient of S1P between the lymph and the lymphoid tissue suggests that S1P1 facilitates directional movement of cells into the lymph (Pappu et al., 2007; Schwab et al., 2005). S1P has been shown to promote directional migration in a number of cells *in vitro*, including lymphocytes(Graeler and Goetzl, 2002; Matloubian et al., 2004; Spiegel and Milstien, 2003). Stimulation of S1P1 has also been shown to trigger down-stream pathways involved in cell migration such as Gαi-coupling,

Rac activation, cortactin translocation, and actin polymerization (Lee et al., 2006; Nombela-Arrieta et al., 2007; Spiegel and Milstien, 2003).

We propose a model that unifies these findings for the role of lymphocye S1P1 during egress. S1P1 transduces a chemoattractant signal for cell migration into the lymph that directly competes for cell attention away from the retention chemoattratant cues emanating from the lymphoid tissue. In a given lymphocyte undergoing egress, if the S1P1 signal is stronger, the outcome will be cell movement into the lymphatic compartment; whereas, retention signal domination will result in the cell staying in the lymphoid tissue. Competition between overlapping Gαi-coupled chemoattactant cues determining the outcome of cell migration has been observed previously for neutrophil migration *in vitro* (Foxman et al., 1997; Foxman et al., 1999; Heit et al., 2002). One important question arising from this model for S1P1 function is how the competition between egress-promoting signal and retention signals is mediated at the molecular level. Again, observations made of neutrophil migration *in vitro* may be instructive for speculation about the molecular basis for the interplay between S1P1 and retention signals in lymphocytes during egress (Heit et al., 2002). Two models could be considered. In a "quantitative model" S1P1 egress-promoting and CCR7 retention signals trigger the same down-stream pathway such as the PI3K pathway, as in the case of IL-8 and LTB4 signals in neutrophils (Heit et al., 2002). As the signaling output is not unique, the resulting dominating signal is one that is quantitatively stronger. Factors that determine the strength of a GPCR signal may include the amount of receptor, ligand availability, ligand

affinity, g-protein coupling efficiency, and amounts of modulators of G-protein signaling such as RGS. Consistent with this quantitative model, we have found the efficiency of T lymphocyte egress from lymph nodes is highly sensitive to the amount of S1P1 and CCR7 (Lo et al., 2005; Pham et al., 2008). Genetic hemizygosity of S1P1 causes reduced T cell egress efficiency, resulting in a 2-3 fold reduction of CD4+ T lymphocytes in the lymph and blood (Pham et al., 2008). To vary the level of surface S1P1 over a wider range and correlate these S1P1 levels to lymphocyte egress, we treated mice with multiple doses of FTY720. In these experiments, we observed a tight relationship between the extent of surface S1P1 down-modulation induced by FTY720 and egress inhibition, as measured by the loss of T cells in the blood and lymph, over four different levels of surface S1P1(Pham et al., 2008). Similarly, a two-fold decrease or increase of CCR7 in heterozygous or transgenic T cells led to enhanced and reduced egress, respectively (Pham et al., 2008). In an alternative "qualitative model," S1P1 egress-promoting and CCR7 retention signals activate two different down-stream pathways, such as a p38 MAPK and a PI3K pathway, for example (Heit et al., 2002). The stronger, "winning" signal is qualitatively dominant to the other signal such that when there is sufficient amount of the dominant signal, the sub-dominant signal is "turned off." Such hierarchical relationship between two Gαi-coupled chemoattrant receptors has been observed in neutrophils, where the fMLP/p38 pathway appears to be dominant over the IL-8/PI3K pathway (Heit et al., 2002). The ability of different Gαi-coupled receptors to trigger distinct down-stream signaling pathways might

depend on unique coupling motifs, receptor oligomerization, or differential active state conformations (Drake et al., 2008; Shukla et al., 2008; Whistler et al., 2002). Characterization of the molecular basis for the interplay between S1P1 egress-promoting and retention signals will be an interesting subject for future investigation.

CCR7 has a central role in T lymphocyte homeostasis, mediating cell homing, compartmentalization, and motility within lymphoid organs, as well as transducing CCL19 survival signal (Asperti-Boursin et al., 2007; Forster et al., 1999; Link et al., 2007; Okada and Cyster, 2007; Worbs et al., 2007). The data presented here expand the scope of CCR7 functions further by showing that it has a role in retaining T lymphocytes in lymphoid tissues (Pham et al., 2008). Accumulating evidence indicates lymphocyte CCR7 signaling may be an important point of egress regulation across physiological settings. We have observed down-modulation of CCR7 expression in parallel with up-regulation of S1P1 in newly generated effector cells, suggesting that this shift in relative egress promoting and retention signals might contribute to the timely release of competent effector cells to travel to the sites of infection (Pham et al., 2008). Similarly, down-regulation of CCL21 expression in lymphoid tissues following viral infection might be important for export of T cells into circulation (Mueller et al., 2007). Recent studies find cyclic modulation of CCR7's ligand sensitivity in T lymphocytes, with cells in the blood being more responsive to ligand than those from the lymphoid tissues (Britschgi et al., 2008). These findings imply that T cells that recently entered the CCL19- and CCL21-high environment of lymphoid

organs from the blood will experience the greatest amount of retention signal, which gets down-modulated over time as cells transit in the lymphoid tissues. Thus, CCR7 signaling may play a role in setting the timing for lymphocyte egress during homeostatic recirculation. One implication of the identification of CCR7 as lymphocyte retention factor is that entry defect might not sufficiently account for the reduced naïve and regulatory T cells in lymphoid tissues of CCR7-deficient mice and that alteration of lymphocyte egress needs to be taken into account. Consequentially, the profound defect of immune suppressive function of $CRR7^{-/-}$ T-reg *in vivo* might be explained in part by the disregulation of egress of these cells (Forster et al., 2008; Schneider et al., 2007). Similarly, the delayed clearance of MHV-68 infection in the lungs of $CCR7^{-/-}$ mice may be due in part to pre-mature release of effector cells, which are yet to fully develop their effector competency, from lymphoid tissues (Kocks et al., 2009).

With CCR7 as a prototype, our studies have identified a new class of factors that negatively regulates lymphocyte egress: Gαi-coupled retention receptors. More importantly, our data implicate existence of one or more additional Gαi-coupled retention receptors in T cells that remain to be identified (Pham et al., 2008). B cells are also retained by Gαi-mediated signals, but no receptor has been accounted for this effect. We have initiated studies to determine if the Gαi-coupled receptors CXCR4 and CXCR5 play a role in B cell retention. Preliminary analyses have not established a significant role of either receptor alone. Future studies involving combined inactivation of both CXCR4 and CXCR5 signaling may be able to determine whether these receptors have

retention effects in B cells. It is worth noting that T and B cell interstitial motility has also been shown to be dependent on Gαi-signaling (Han et al., 2005; Huang et al., 2007; Hwang et al., 2007; Okada and Cyster, 2007). T and B cells lacking Gαi2, one of the two PTX-sensitive Gα proteins most abundantly expressed in lymphocytes, exhibit 40% and 20% reduction in motility, respectively. PTX-treatment is found to reduce T cell motility by 40%-60%. In T cells, up to 40% of this Gαi-dependent motility can be accounted for by CCR7. The Gαi-coupled receptors responsible for the remaining T cell and all B cell Gαi-dependent motility remain to be characterized. Future efforts to discover additional Gαi-coupled receptors involved in lymphocyte retention may result in identification of additional Gαi-coupled receptors that mediate motility in these cells.

Traditionally, lymphocytes were thought to egress from lymph nodes by exiting into the medullary lymphatic sinuses, where cells could flow into the efferent lymph and be carried out of the organs (Cyster, 2005; Picker, 1999; Wei et al., 2005a). However, ultra-structural studies since the late 1960's have indicated the existence of sinus-like structures in the paracortical area of the lymph nodes that are full of lymphocytes and appear to connect to the medullary sinuses (Belisle and Sainte-Marie, 1981; Kelly, 1975; Soderstrom and Stenstrom, 1969). To further characterize these paracortical (or cortical) sinuses and their role in lymphocyte trafficking, we stained lymph node sections with the lymphatic marker LYVE-1 and found that these sinusoidal structures stained positively for this marker (Pham et al., 2008). Consistent with the idea that cortical sinuses are sites for lymphocyte egress, we also found that these structures contain an

abundant number of T and B cells, but not dendritic cells, which are not known to have significant egress activity. More conclusively, we provided the first functional evidence for the role of cortical sinuses in lymphocyte egress by showing that S1P1$^{-/-}$ T cells fail to accumulate inside these structures. Our findings on cortical sinuses have been extended further by recent *in vivo* imaging studies, in which T cells appear to undergo S1P1-dependent entry into LYVE-1+ cortical sinuses and get carried by flow toward the medulla during egress (Grigorova et al., 2009). Cortical sinuses have also been demonstrated to support the egress of B cells from lymph nodes (Sinha et al., 2009).

Our competing-cue model for lymphocyte egress suggests that lymphocytes undergoing egress from the lymph nodes would encounter S1P and CCR7 signal in a common environment, a situation that appears to be true at the cortical sinuses. Staining for CCL21, CCR7's ligand, shows that the ligand availability is abundant in the deep T zone and extends, though at a lesser level, to the edge of the egress barrier (Pham et al., 2008). Lymphatic endothelium itself is coated with CCL21 (Cueni and Detmar, 2006). S1P level, on the other hand, is high in the lymph and low in the lymph nodes and recent evidence suggest this S1P gradient extends beyond the endothelial border into the lymph node parenchyma. First, CCR7 deficient T cells, which are unable to localize in the deep T zone and displaced toward lymphatic sinuses, have less surface S1P1 as detected by flow cytometric analysis (Pham et al., 2008). Secondly, in immunoflourescence studies B cells localized further away from cortical sinuses have more immuno-reactivity against an anti-S1P1 antibody, as compared to

those localized closer to the sinuses (Sinha et al., 2009). Since, S1P1 is known to be highly sensitive to S1P-induced internalization (Schwab et al., 2005), these findings indicate that lymphocytes are exposed to an increased amount of S1P as they move from the deep lymph node parenchyma toward the lymphatics. Whether lymphocytes undergoing egress at the medullary sinuses experience similar overlapping gradients of egress-promoting and retention signals as at the cortical sinuses has not been demonstrated. We speculate that such interplay of competing chemoattractant cues also takes place in the medulla and might involve yet to be identified retention signals.

Another advance that our studies have made toward understanding mechanisms regulating lymphocyte egress is the identification of LECs as the source of lymph S1P required for egress from lymph nodes and Peyer's patches. We had hypothesized that lymph S1P is produced locally by the endothelial cells lining the lymphatic sinuses. Our hypothesis was based on the following observations (Pappu et al., 2007b): 1) Mx-CRE mediated deletion of *Sphk1* in mice lacking *Sphk2* results in a loss of lymph S1P; 2) LECs exhibit efficient Mx-CRE mediated recombination of *LoxP*-flanked genes; 3) The source of lymph S1P is radiation-resistant. To directly test this hypothesis, we generated mice which, in addition to lacking *Sphk2*, have *Sphk1* conditionally ablated by activity of a CRE expressed under the *Lyve-1* locus. In these lymphatic *Sphk*-deficient (or *Lyve-1 Cre Sphk*-deficient) mice, there is a marked reduction of lymph S1P and a block of lymphocyte egress from the lymph nodes and Peyer's patches. Using *LoxP*-STOP-*LoxP* Rosa26YFP reporter mice, we found that deletion of

LoxP flanked genes in mice carrying the *Lyve-1 Cre* knockin gene is restricted to LECs, a fraction of BECs, and LYVE-1+ macrophages, a pattern that reflects the embryonic and adult expression of LYVE-1 (Gordon et al., 2008; Makinen et al., 2007). The loss of lymph S1P, however, appears to be due to loss of *Sphk-1* and *Sphk-2* expression in LECs. We demonstrated that blood and lymph S1P were maintained by separate pools, as lymphatic *Sphk*-deficient mice had a loss of lymph S1P but exhibited normal blood S1P, consistent with findings in previous studies using *Mx-Cre Sphk*-deficient mice (Pappu et al., 2007). Using bone marrow chimera studies, we also established that LYVE-1+ macrophages made no significant contribution to lymph S1P. Although we have yet to demonstrate that the lymph S1P needed for induction of lymphocyte egress is produced locally within the lymph nodes and at the exit sites, we believe this is likely the case. In two-photon imaging studies, cells are observed to migrate into the lymphatic sinuses in explanted lymph nodes that have been removed hours before from mice (Grigorova et al., 2009; Wei et al., 2005). Since the half-life of plasma S1P has been demonstrated to be on the order of 15'-20' (Venkataraman et al., 2008), the imaging observations suggest that S1P is probably continually produced in explanted lymph nodes to support lymphocyte egress into the lymphatics. Future experiments in which lymph nodes from *Lyve-1 Cre Sphk-deficient* mice are transplanted into wild-type hosts might demonstrate more conclusively the local production of S1P for lymphocyte egress.

When we further analyzed the egress defect phenotype in the *Lyve-1 Cre Sphk*-deficient mice using histological analyses, we found that LYVE-1+ cortical

sinuses appeared collapsed and lacked lymphocytes. This observation raises two questions: 1) Is the egress block due to the lack of S1P acting on lymphocyte S1P1 to induce egress promoting signal or on the lymphatic endothelium to keep it permissive for lymphocyte transmigration into the lymph; 2) Is the egress block due to developmental defect of lymphatic sinuses? To address these issues, we tested whether treatment of the lymphocytes with PTX to inactivate Gαi-mediated retention signals would restore lymphocyte egress in *Lyve-1 Cre Sphk*-deficient mice. Indeed, we found that PTX treatment not only led to recovery of lymphocytes in the lymph of mice lacking lymph S1P, but also restored cell localization within cortical sinus exit structures. These findings suggest that the lack of lymph S1P to act on lymphocyte S1P1 to overcome retention signals is the mechanism underlying the egress defect in lymphatic *Sphk*-deficient mice and that the lymphatic vasculature in these mice can support efficient cell migration during lymphocyte egress.

Because of the established functions of S1P signaling in the development of the blood vasculature (Kono et al., 2008), we sought to determine if ablation of lymph S1P had an effect on lymphatic development. Intriguingly, we have observed alteration of lymphatic vessel architecture in lymphatic *Sphk*-deficient mice, providing evidence for the first time that S1P signaling may have a role in physiological development of the lymphatic system. When examining lymphatic morphology of the *Lyve-1 Cre Sphk*-deficient mice using the established model of trachea and diaphragm afferent lymphatics (Baluk et al., 2007), we find that the lymphatic vessels in these mice appeared tortuous and ragged, with “button”-like

junctions at the initial lymphatics being less defined and made up of fewer "buttons," as compared to the vessels in control mice. Despite these differences, lymphatic functions in fluid absorption and cell migration appear to be preserved in lymphatic *Sphk*-deficient mice, which survive to adulthood and show no apparent lymphedema. We have demonstrated that efficient lymphocyte egress can occur at the efferent lymphatic in these mice. Furthermore, we have observed normal number of skin-derived dendritic cells in the skin-draining lymph nodes of lymphatic *Sphk*-deficient mice in steady state conditions, suggesting that the afferent lymphatics of these mice can support leukocyte homing to lymph nodes from peripheral tissues to a similar extent as those in control mice (Unpublished observations, Pham THM and Cyster JG). These are early observations on a potential role of S1P signaling in lymphatic development and raise many questions. First, are the lymphatic structural alterations seen direct effects of the lack of lymph S1P signaling on LECs or indirect effects? What are the molecular mechanisms of S1P signaling in lymphatic development? What are the physiological consequences of these alterations in steady state conditions or other conditions? Human lymphatic endothial cells (HLECs) derived from dermal tissues(Yoon et al., 2008), as well as LECs isolated from mouse lymph nodes in our studies (Pham TMP & Cyster JG, unpublished observations), express S1P1 and, to a lesser extent, S1P3, suggesting that LECs are capable of transducing signals from extracellular S1P. *In vitro*, S1P stimulates cell migration, tubular formation, and exocytosis of Angiopoietin-2 in HLECs in a S1P1, Gαi, PLC, and Ca^{2+} dependent manner (Jang et al., 2009;

Yoon et al., 2008). S1P has been shown to induce lymphangiogenesis in matrigel plug assays *in vivo*, and major lymphatic defects have been demonstrated in mice lacking Angiopoietin-2(Gale et al., 2002; Yoon et al., 2008). In blood endothelial cells, S1P stimulation leads to localization of VE-Cadherin toward cell-cell junctions (Lee et al., 1999), a cellular mechanism that might explain the disregulation of VE-Cadherin patterning seen in *Lyve-1 Cre Sphk*-deficient mice. Future studies using mice lacking S1P-receptors specifically in LECs will allow further understanding on how S1P signaling regulates lymphatic development.

Finally, our data from the studies presented here have contributed to the resolution of the debate on whether S1P/S1P1 signaling acts on lymphocytes or endothelial cells to regulate lymphocyte egress (Cahalan and Parker, 2008; Rosen and Goetzl, 2005; Rosen et al., 2007; Schwab and Cyster, 2007). Using S1P agonists to perturb lymphocyte egress, it has been argued that action of S1P and its agonists on endothelial S1P receptors leads to tightening of the lymphatic endothelial barrier to prevent lymphocyte exit (Mandala et al., 2002; Sanna et al., 2006; Wei et al., 2005). While there have been examples of S1P signaling in endothelial cells causing junctional changes and affecting endothelial barrier integrity (Marsolais et al., 2009), whether this type of mechanism is at the center of lymphocyte regulation is neither well nor directly demonstrated. Indeed, in the studies used to support a LEC-centric model for how S1P signaling affects lymphocyte egress, the experimental manipulations are not specific to endothelial cells and direct observations of the changes within the endothelial are lacking

(Mandala et al., 2002; Sanna et al., 2006; Wei et al., 2005). Our data showing PTX treatment of the lymphocyte restores egress in lymphocytes exposed to FTY720 are consistent with the conclusion that the drug acts to down-modulate S1P1 function, rendering cells unable to overcome Gαi-mediated retention to undergo egress. When these retention signals are removed, cells can egress again (Pham et al., 2008). In mice that lack lymph S1P, we have shown that lymphocyte egress block can also be reversed by PTX treatment of the lymphocytes, again suggesting that S1P signaling operates mainly through the lymphocytes to promote egress. Together with data obtained from other studies, the case for S1P/S1P1 signaling acting to promote lymphocyte egress through a lymphocyte intrinsic mechanism is compelling (Lo et al., 2005; Matloubian et al., 2004; Pappu et al., 2007; Pham et al., 2008; Schwab et al., 2005; Shiow et al., 2006): 1) S1P1 is required intrinsically within the lymphocytes for egress; 2) Loss of lymphocyte S1P1 function due to genetic deficiency, exposure to FTY720, and activation results in egress block, which can be reversed by inhibition of Gαi-mediated retention signals, overexpression of S1P1, and CD69 deficiency within the lymphocytes; 3) When the lack of lymph S1P leads to a loss of positive egress promoting signal and a egress block, antagonizing Gαi retention signals within the lymphocytes also restore lymphocyte egress.

In summary, we have provided evidence to support a model for lymphocyte egress from the lymph nodes whereby lymphatic endothelial cells produce lymph S1P, which acts directly on lymphocyte S1P1 to overcome Gαi-mediated retention signals to promote egress. Thus, a plausible mechanism by

which S1P agonists, which are being developed for clinical use (Massberg and von Andrian, 2006), exert their immunosuppressive activity is to down-modulate lymphocyte S1P1 functions.

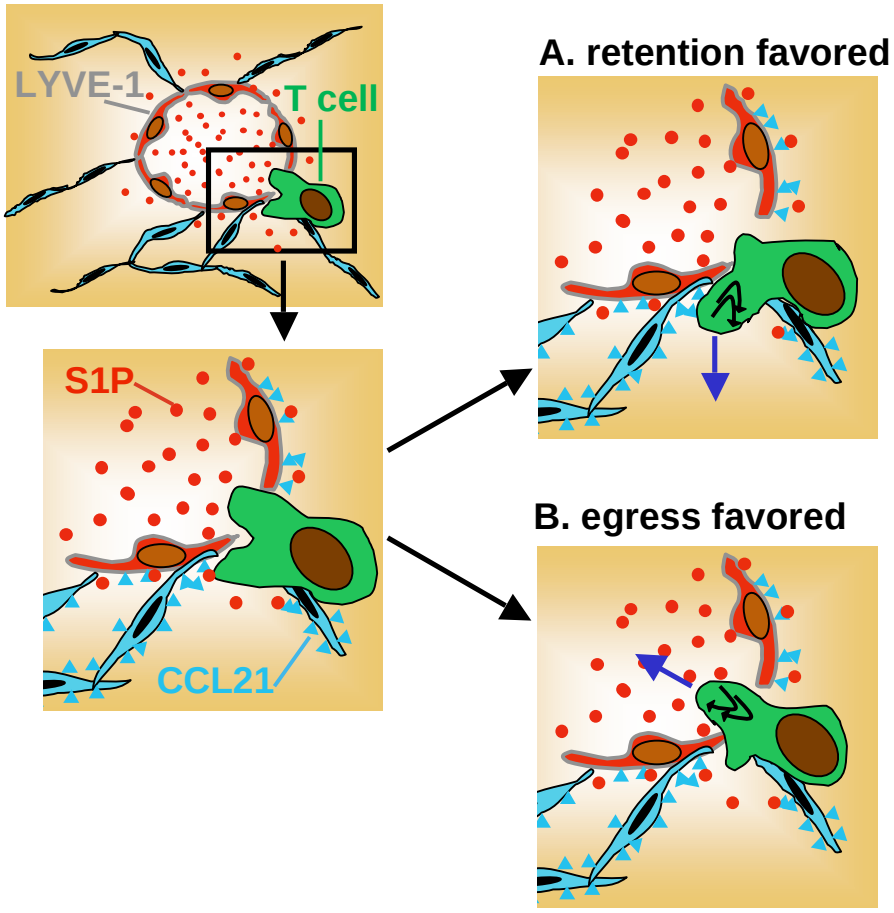


Figure 1. Proposed model for $S1P_1$ function during the early steps of T cell egress from lymph nodes. A T cell (green) is diagrammed migrating upon a $CCL21^+$ (turquoise triangle) T zone stromal cell network and encountering an S1P-containing (red circles) $LYVE\text{-}1^+$ cortical sinusoid (gray), made up of S1P-producing lymphatic endothelial cells. Both compartments are crowded with other lymphocytes (not depicted). The T cell expresses both CCR7 and $S1P_1$ (not shown) and is illustrated simultaneously encountering CCL21 and S1P. Two possible outcomes can result. (A) The CCR7 retention signal dominates and the cell makes a committed pseudopod away from the LYVE-1+ cortical sinusoid. This outcome may be favored with T cells that have reduced $S1P_1$ due to desensitization, functional down-modulation or genetic deficiency, or having more CCR7 due to transgenesis. (B) The $S1P_1$ egress-promoting signal dominates, leading the cell to make a committed pseudopod into the cortical sinusoid. This outcome may be favored in a T cell that has been in the lymph node for several hours and has fully upregulated $S1P_1$, or that has no or reduced levels of CCR7 due to genetic deficiency, desensitization or activation-induced down-modulation.

REFERENCES

Allende, M.L., Dreier, J.L., Mandala, S., and Proia, R.L. (2004a). Expression of the sphingosine-1-phosphate receptor, S1P1, on T-cells controls thymic emigration. Journal of Biological Chemistry *279*, 15396-15401.

Allende, M.L., Sasaki, T., Kawai, H., Olivera, A., Mi, Y., van Echten-Deckert, G., Hajdu, R., Rosenbach, M., Keohane, C.A., Mandala, S*., et al.* (2004b). Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. J Biol Chem *279*, 52487-52492.

Arnold, C.N., Butcher, E.C., and Campbell, D.J. (2004). Antigen-Specific Lymphocyte Sequestration in Lymphoid Organs: Lack of Essential Roles for alpha(L) and alpha(4) Integrin-Dependent Adhesion or Galpha(i) Protein-Coupled Receptor Signaling. Journal of Immunology *173*, 866-873.

Asperti-Boursin, F., Real, E., Bismuth, G., Trautmann, A., and Donnadieu, E. (2007). CCR7 ligands control basal T cell motility within lymph node slices in a phosphoinositide 3-kinase-independent manner. J Exp Med *204*, 1167-1179.

Azzali, G. (2003). Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. Immunological reviews *195*, 178-189.

Bajenoff, M., Egen, J.G., Koo, L.Y., Laugier, J.P., Brau, F., Glaichenhaus, N., and Germain, R.N. (2006). Stromal cell networks regulate lymphocyte entry, migration and territoriality in lymph nodes. Immunity *doi:10.1016/j.immuni.2006.10.011*.

Bajenoff, M., Glaichenhaus, N., and Germain, R.N. (2008). Fibroblastic reticular cells guide T lymphocyte entry into and migration within the splenic T cell zone. J Immunol *181*, 3947-3954.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E., and McDonald, D.M. (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med *204*, 2349-2362.

Belisle, C., and Sainte-Marie, G. (1981). Tridimensional study of the deep cortex of the rat lymph node. III. Morphology of the deep cortex units. Anatomical Record *199*, 213-226.

Beltman, J.B., Maree, A.F., Lynch, J.N., Miller, M.J., and de Boer, R.J. (2007). Lymph node topology dictates T cell migration behavior. J Exp Med *204*, 771-780.

Bohler, T., Waiser, J., Schuetz, M., Neumayer, H.H., and Budde, K. (2004). FTY720 exerts differential effects on CD4+ and CD8+ T-lymphocyte subpopulations expressing chemokine and adhesion receptors. Nephrol Dial Transplant *19*, 702-713.

Britschgi, M.R., Link, A., Lissandrin, T.K., and Luther, S.A. (2008). Dynamic modulation of CCR7 expression and function on naive T lymphocytes in vivo. J Immunol *181*, 7681-7688.

Cahalan, M.D., and Parker, I. (2008). Choreography of cell motility and interaction dynamics imaged by two-photon microscopy in lymphoid organs. Annu Rev Immunol *26*, 585-626.

Carlson, C.M., Endrizzi, B.T., Wu, J., Ding, X., Weinreich, M.A., Walsh, E.R., Wani, M.A., Lingrel, J.B., Hogquist, K.A., and Jameson, S.C. (2006). Kruppel-like factor 2 regulates thymocyte and T-cell migration. Nature *442*, 299-302.

Chiba, K. (2005). FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. Pharmacology & therapeutics *108*, 308-319.

Chiba, K., Yanagawa, Y., Masubuchi, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M., and Hoshino, Y. (1998). FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J Immunol *160*, 5037-5044.

Cueni, L.N., and Detmar, M. (2006). New insights into the molecular control of the lymphatic vascular system and its role in disease. The Journal of investigative dermatology *126*, 2167-2177.

Cyster, J.G. (2005). Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol *23*, 127-159.

Drake, M.T., Violin, J.D., Whalen, E.J., Wisler, J.W., Shenoy, S.K., and Lefkowitz, R.J. (2008). beta-arrestin-biased agonism at the beta2-adrenergic receptor. J Biol Chem *283*, 5669-5676.

Forster, R., Davalos-Misslitz, A.C., and Rot, A. (2008). CCR7 and its ligands: balancing immunity and tolerance. Nature reviews *8*, 362-371.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23-33.

Foxman, E.F., Campbell, J.J., and Butcher, E.C. (1997). Multistep navigation and the combinatorial control of leukocyte chemotaxis. The Journal of cell biology *139*, 1349-1360.

Foxman, E.F., Kunkel, E.J., and Butcher, E.C. (1999). Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation. Journal of Cell Biology *147*, 577-588.

Fujita, T., Hirose, R., Yoneta, M., Sasaki, S., Inoue, K., Kiuchi, M., Hirase, S., Chiba, K., Sakamoto, H., and Arita, M. (1996). Potent immunosuppressants, 2-alkyl-2-aminopropane-1,3-diols. Journal of medicinal chemistry *39*, 4451-4459.

Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Chiba, K., Hoshino, Y., and Okumoto, T. (1994). Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. The Journal of antibiotics *47*, 208-215.

Gale, N.W., Thurston, G., Hackett, S.F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M.H., Jackson, D*., et al.* (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Developmental cell *3*, 411-423.

Gordon, E.J., Gale, N.W., and Harvey, N.L. (2008). Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE-1 is also expressed on embryonic blood vessels. Dev Dyn *237*, 1901-1909.

Gowans, J.L. (1996). The lymphocyte--a disgraceful gap in medical knowledge. Immunology today *17*, 288-291.

Gowans, J.L., and Knight, E.J. (1964). The Route of Re-Circulation of Lymphocytes in the Rat. Proc R Soc Lond B Biol Sci *159*, 257-282.

Graeler, M., and Goetzl, E.J. (2002). Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. Faseb J *16*, 1874-1878.

Grigorova, I.L., Schwab, S.R., Phan, T.G., Pham, T.H., Okada, T., and Cyster, J.G. (2009). Cortical sinus probing, S1P1-dependent entry and flow-based capture of egressing T cells. Nat Immunol *10*, 58-65.

Han, S.B., Moratz, C., Huang, N.N., Kelsall, B., Cho, H., Shi, C.S., Schwartz, O., and Kehrl, J.H. (2005). Rgs1 and Gnai2 regulate the entrance of B lymphocytes into lymph nodes and B cell motility within lymph node follicles. Immunity *22*, 343-354.

Heit, B., Tavener, S., Raharjo, E., and Kubes, P. (2002). An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. Journal of Cell Biology *159*, 91-102.

Henning, G., Ohl, L., Junt, T., Reiterer, P., Brinkmann, V., Nakano, H., Hohenberger, W., Lipp, M., and Forster, R. (2001). CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. J Exp Med *194*, 1875-1881.

Huang, A.Y., Qi, H., and Germain, R.N. (2004). Illuminating the landscape of in vivo immunity: insights from dynamic in situ imaging of secondary lymphoid tissues. Immunity *21*, 331-339.

Huang, J.H., Cardenas-Navia, L.I., Caldwell, C.C., Plumb, T.J., Radu, C.G., Rocha, P.N., Wilder, T., Bromberg, J.S., Cronstein, B.N., Sitkovsky, M.*, et al.* (2007). Requirements for T lymphocyte migration in explanted lymph nodes. Journal of Immunology *178*, 7747-7755.

Hwang, I.Y., Park, C., and Kehrl, J.H. (2007). Impaired trafficking of Gnai2+/- and Gnai2-/- T lymphocytes: implications for T cell movement within lymph nodes. J Immunol *179*, 439-448.

Jang, C., Koh, Y.J., Lim, N.K., Kang, H.J., Kim, D.H., Park, S.K., Lee, G.M., Jeon, C.J., and Koh, G.Y. (2009). Angiopoietin-2 exocytosis is stimulated by sphingosine-1-phosphate in human blood and lymphatic endothelial cells. Arteriosclerosis, thrombosis, and vascular biology *29*, 401-407.

Karpanen, T., and Alitalo, K. (2008). Molecular biology and pathology of lymphangiogenesis. Annual review of pathology *3*, 367-397.

Kelly, R.H. (1975). Functional anatomy of lymph nodes. I. The paracortical cords. International Archives of Allergy and Applied Immunology *48*, 836-849.

Khanna, K.M., and Lefrancois, L. (2008). Geography and plumbing control the T cell response to infection. Immunol Cell Biol *86*, 416-422.

Kharel, Y., Lee, S., Snyder, A.H., Sheasley-O'neill S, L., Morris, M.A., Setiady, Y., Zhu, R., Zigler, M.A., Burcin, T.L., Ley, K.*, et al.* (2005). Sphingosine kinase 2 is required for modulation of lymphocyte traffic by FTY720. J Biol Chem *280*, 36865-36872.

Kihara, A., and Igarashi, Y. (2008). Production and release of sphingosine 1-phosphate and the phosphorylated form of the immunomodulator FTY720. Biochimica et biophysica acta *1781*, 496-502.

Kocks, J.R., Adler, H., Danzer, H., Hoffmann, K., Jonigk, D., Lehmann, U., and Forster, R. (2009). Chemokine receptor CCR7 contributes to a rapid and efficient clearance of lytic murine gamma-herpes virus 68 from the lung, whereas bronchus-associated lymphoid tissue harbors virus during latency. J Immunol *182*, 6861-6869.

Kono, M., Allende, M.L., and Proia, R.L. (2008). Sphingosine-1-phosphate regulation of mammalian development. Biochimica et biophysica acta *1781*, 435-441.

Lee, J.F., Ozaki, H., Zhan, X., Wang, E., Hla, T., and Lee, M.J. (2006). Sphingosine-1-phosphate signaling regulates lamellipodia localization of cortactin complexes in endothelial cells. Histochemistry and cell biology *126*, 297-304.

Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell *99*, 301-312.

Link, A., Vogt, T.K., Favre, S., Britschgi, M.R., Acha-Orbea, H., Hinz, B., Cyster, J.G., and Luther, S.A. (2007). Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. Nat Immunol *8*, 1255-1265.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J*., et al.* (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. The Journal of clinical investigation *106*, 951-961.

Lo, C.G., Xu, Y., Proia, R.L., and Cyster, J.G. (2005). Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J Exp Med *201*, 291-301.

Makinen, T., Norrmen, C., and Petrova, T.V. (2007). Molecular mechanisms of lymphatic vascular development. Cell Mol Life Sci *64*, 1915-1929.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C*., et al.* (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science *296*, 346-349.

Marsolais, D., Hahm, B., Walsh, K.B., Edelmann, K.H., McGavern, D., Hatta, Y., Kawaoka, Y., Rosen, H., and Oldstone, M.B. (2009). A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection. Proceedings of the National Academy of Sciences of the United States of America *106*, 1560-1565.

Massberg, S., and von Andrian, U.H. (2006). Fingolimod and sphingosine-1-phosphate--modifiers of lymphocyte migration. The New England journal of medicine *355*, 1088-1091.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

Miller, M.J., Wei, S.H., Parker, I., and Cahalan, M.D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science *296*, 1869-1873.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G.F., Spiegel, S., and Proia, R.L. (2005). Essential role for sphingosine kinases in neural and vascular development. Molecular and cellular biology *25*, 11113-11121.

Mueller, S.N., and Ahmed, R. (2008). Lymphoid stroma in the initiation and control of immune responses. Immunological reviews *224*, 284-294.

Mueller, S.N., Hosiawa-Meagher, K.A., Konieczny, B.T., Sullivan, B.M., Bachmann, M.F., Locksley, R.M., Ahmed, R., and Matloubian, M. (2007). Regulation of homeostatic chemokine expression and cell trafficking during immune responses. Science *317*, 670-674.

Nombela-Arrieta, C., Mempel, T.R., Soriano, S.F., Mazo, I., Wymann, M.P., Hirsch, E., Martinez, A.C., Fukui, Y., von Andrian, U.H., and Stein, J.V. (2007). A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. J Exp Med *204*, 497-510.

Okada, T., and Cyster, J.G. (2007). CC Chemokine Receptor 7 Contributes to Gi-Dependent T Cell Motility in the Lymph Node. Journal of Immunology *178*, 2973-2978.

Oliver, G., and Srinivasan, R.S. (2008). Lymphatic vasculature development: current concepts. Annals of the New York Academy of Sciences *1131*, 75-81.

Pappu, R., Schwab, S.R., Cornelissen, I., Pereira, J.P., Regard, J.B., Xu, Y., Camerer, E., Zheng, Y.W., Huang, Y., Cyster, J.G., and Coughlin, S.R. (2007). Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science *316*, 295-298.

Pham, T.H., Okada, T., Matloubian, M., Lo, C.G., and Cyster, J.G. (2008). S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. Immunity *28*, 122-133.

Picker, L.J.S., M.H. (1999). Fundamental Immunology. *4*, 479-531.

Podgrabinska, S., Braun, P., Velasco, P., Kloos, B., Pepper, M.S., and Skobe, M. (2002). Molecular characterization of lymphatic endothelial cells. Proceedings of the National Academy of Sciences of the United States of America *99*, 16069-16074.

Rosen, H., Alfonso, C., Surh, C.D., and McHeyzer-Williams, M.G. (2003). Rapid induction of medullary thymocyte phenotypic maturation and egress inhibition by nanomolar sphingosine 1-phosphate receptor agonist. Proceedings of the National Academy of Sciences of the United States of America *100*, 10907-10912.

Rosen, H., and Goetzl, E.J. (2005). Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nature reviews *5*, 560-570.

Rosen, H., Sanna, M.G., Cahalan, S.M., and Gonzalez-Cabrera, P.J. (2007). Tipping the gatekeeper: S1P regulation of endothelial barrier function. Trends in immunology *28*, 102-107.

Sanna, M.G., Wang, S.K., Gonzalez-Cabrera, P.J., Don, A., Marsolais, D., Matheu, M.P., Wei, S.H., Parker, I., Jo, E., Cheng, W.C.*, et al.* (2006). Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P(1) antagonist in vivo. Nat Chem Biol *2*, 434-441.

Schneider, M.A., Meingassner, J.G., Lipp, M., Moore, H.D., and Rot, A. (2007). CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. J Exp Med *204*, 735-745.

Schwab, S.R., and Cyster, J.G. (2007). Finding a way out: lymphocyte egress from lymphoid organs. Nat Immunol *8*, 1295-1301.

Schwab, S.R., Pereira, J.P., Matloubian, M., Xu, Y., Huang, Y., and Cyster, J.G. (2005). Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science *309*, 1735-1739.

Shiow, L.R., Rosen, D.B., Brdickova, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G., and Matloubian, M. (2006). CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature *440*, 540-544.

Shukla, A.K., Violin, J.D., Whalen, E.J., Gesty-Palmer, D., Shenoy, S.K., and Lefkowitz, R.J. (2008). Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors. Proceedings of the National Academy of Sciences of the United States of America *105*, 9988-9993.

Sinha, R.K., Park, C., Hwang, I.Y., Davis, M.D., and Kehrl, J.H. (2009). B lymphocytes exit lymph nodes through cortical lymphatic sinusoids by a mechanism independent of sphingosine-1-phosphate-mediated chemotaxis. Immunity *30*, 434-446.

Soderstrom, N., and Stenstrom, A. (1969). Outflow paths of cells from the lymph node parenchyma to the efferent lymphatics--observations in thin section histology. Scandinavian Journal of Haematology *6*, 186-196.

Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol *4*, 397-407.

Venkataraman, K., Lee, Y.M., Michaud, J., Thangada, S., Ai, Y., Bonkovsky, H.L., Parikh, N.S., Habrukowich, C., and Hla, T. (2008). Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circulation research *102*, 669-676.

von Andrian, U.H., and Mempel, T.R. (2003). Homing and cellular traffic in lymph nodes. Nature Reviews. Immunology *3*, 867-878.

Wei, S.H., Rosen, H., Matheu, M.P., Sanna, M.G., Wang, S.K., Jo, E., Wong, C.H., Parker, I., and Cahalan, M.D. (2005a). Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. Nature immunology *6*, 1228-1235.

Whistler, J.L., Gerber, B.O., Meng, E.C., Baranski, T.J., von Zastrow, M., and Bourne, H.R. (2002). Constitutive activation and endocytosis of the complement factor 5a receptor: evidence for multiple activated conformations of a G protein-coupled receptor. Traffic (Copenhagen, Denmark) *3*, 866-877.

Worbs, T., Mempel, T.R., Bolter, J., von Andrian, U.H., and Forster, R. (2007). CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. Journal of Experimental Medicine *204*, 489-495.

Yagi, H., Kamba, R., Chiba, K., Soga, H., Yaguchi, K., Nakamura, M., and Itoh, T. (2000). Immunosuppressant FTY720 inhibits thymocyte emigration. European journal of immunology *30*, 1435-1444.

Yoon, C.M., Hong, B.S., Moon, H.G., Lim, S., Suh, P.G., Kim, Y.K., Chae, C.B., and Gho, Y.S. (2008). Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. Blood *112*, 1129-1138.

Zemann, B., Kinzel, B., Muller, M., Reuschel, R., Mechtcheriakova, D., Urtz, N., Bornancin, F., Baumruker, T., and Billich, A. (2006). Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. Blood *107*, 1454-1458.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature

8/20/09

Date