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Naive and memory T cell trafficking in selectin ligand-deficient mice: the role of fucosyltransferase –IV and –VII in the differential migration of T cell populations

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To the Graduate Council:

I am submitting herewith a thesis written by John Robert Harp entitled "Naive and memory T cell trafficking in selectin ligand-deficient mice: the role of fucosyltransferase –IV and –VII in the differential migration of T cell populations." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Thandi M. Onami, Major Professor

We have read this thesis and recommend its acceptance:

Tim E. Sparer, Mark Y. Sangster

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Naive and memory T cell trafficking in selectin ligand-deficient mice: the role of fucosyltransferase –IV and –VII in the differential migration of T cell populations

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John Robert Harp
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Abstract

The correct and timely delivery of immune cells is critical for protection against foreign antigen. In order for cells to access most organs, there are requirements that must be met to facilitate exit from the blood into extravasculature. The initial requirement is selectin-selectin ligand interactions that mediate tethering and rolling to allow shear resistance. For proper selectin-selectin ligand interaction, glycoproteins must be modified by fucosyltransferases –IV and –VII, which adds fucose to an acceptor substrate to form the sialyl-Lewis^X moiety. Using fucosyltransferase –IV and –VII double knockout (FtDKO) mice, we made several novel observations. Our first observation showed increased numbers of naïve T cells in non-lymphoid organs. To support this observation, we blocked chemokine-mediated entry into lymph nodes (LNs) with pertussis toxin and L-selectin mediated entry with anti-CD62L antibody in WT mice. We also treated WT mice with the S1P1 agonist, FTY720, to retain lymphocytes in LNs. Our results suggested that when access to LN is perturbed, lymphocytes accumulate in non-lymphoid organs. Our second observation showed an enrichment of effector/memory T cells in FtDKO LNs. To determine if effector/memory CD8 T cells were retained in LNs, we transferred naïve and memory CD8 T cells into WT mice then treated the recipient mice with anti-CD62L. We found that LN exit rates of naïve and memory CD8 T cells were similar, but slowed as T cell density decreased. To understand if memory CD8 T cells were using selectin ligand independent mechanisms, we transferred naïve and memory CD8 T cells into WT or FtDKO mice. We found

reduced numbers of memory CD8 T cells in LNs, however, their frequency was increased. We explored this result by transferring CFSE labeled memory CD8 T cells. We found that memory CD8 T cells divide more in FtDKO mice compared to WT. These experiments suggested that selectin ligand deficiencies cause increased frequency of effector/memory T cells in LNs due to low density and increased emptiness induced proliferation. Taken together, these findings reveal how selectin ligand deficiencies contribute to T cell accumulation in non-lymphoid organs and elucidate mechanisms of retention in LNs.

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Abbreviations

BALT	Bronchus associated lymphoid tissue
BM	Bone marrow
CLN	Cervical lymph node
FtDKO	Fucosyltransferase –IV and –VII double knockout
HEV	High endothelial venule
ILN	Iliac lymph node
IngLN	Inguinal lymph node
i.p	Intraperitoneal
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph node
MedLN	Mediastinal lymph node
o-NALT	Organized nasal-associated lymphoid tissue
PBMC	Peripheral blood mononuclear cell
PNAd	Peripheral Node Addressin
PTx	Pertussis toxin
S1P1	Sphingosine 1-phosphate receptor
SLO	Secondary lymphoid organ
T_{cm}	Central memory T cell
T_{em}	Effector memory T cell

Chapter 1

Introduction and Literature Review

Introduction

Seminal research conducted by Gowns and colleagues in rats during the early 1960's has paved the way for understanding the importance of cell migration in immunity [1,2]. Concurrent with these observations, Morris was conducting studies in sheep where he and colleagues identified distinct recirculation processes involving the movement of lymphocytes from the blood to lymph nodes via afferent lymphatics [3]. Furthermore, using the ovine model, surgical procedures allowing access to the lymphatic vessels that drained lymph nodes made examining the recirculation of intravenously injected cells through lymph nodes over extended periods of time extremely informative [4,5]. The knowledge gained during these pioneering studies has contributed immensely to the current experimental designs that seek to elucidate the mechanisms associated with leukocyte migration to tissues and organs.

Lymphocytes play an integral role in providing the necessary protection against foreign antigen. Since these cells respond to antigen through direct contact, their migration to sites where the antigen can be found is essential [6]. This process of cell delivery, which has been actively studied in vitro and confirmed in vivo, begins with tethering or capture, followed by rolling, arrest, crawling, and extravasation (Figure 1.1) [7,8]. The ability for cells to employ these steps and be delivered to the precise organ relies heavily on the expression of various proteins found on the surface of both the adherent cell and the endothelial cell [9]. During an immune response, inflammatory mediators induce changes to endothelium and leukocytes, such that those changes

facilitate entry into peripheral organs at greater frequency [10]. With this greater frequency of trafficking, responses can be initiated and antigens cleared. Understanding the mechanisms that contribute to the delivery of cells during homeostasis or inflammation may provide us with additional insight into therapeutic or diagnostic strategies that could prevent or curtail the effects of misguided trafficking.

Cellular Adhesion

Very specialized vascular endothelium controls the movement of cells from blood into adjacent organs. Typically, cells that migrate to extravascular sites use post capillary venues, but may also be subject to capillary diameter restrictions that allow entry into various organs (i.e. lung) [11,12,13]. During the short time leukocytes are in the blood, blood flow moves leukocytes around rapidly [9]. As a consequence, shear forces move cells through the vasculature at high rates and only those cells with proper attachment can adhere to the vascular endothelium. To achieve proper attachment, leukocytes must employ adhesion molecules to form stable interactions with the vascular wall leading to entry of specific cells while excluding others [14,15]. One location where this specific process occurs frequently is at the lymph node where specialized endothelial cells make up the high endothelial venue. The high endothelial venue supports the trafficking of cells that contain the necessary adhesion molecules that allows attachment to this location. However, endothelial cells in other locations that

experience inflammatory mediators (i.e. TNFa, IFNg, and IL-1) can also upregulate the necessary adhesion molecules that allow migration to locations where in the steady state, cellular access is less likely to occur [16].

Selectins

In order to reach extravascular organs, leukocytes must interact with several adhesion molecules. The first and most important step for successful exit from the vasculature is initial tethering and rolling which is facilitated by selectins. Selectins are c-type lectins expressed on leukocytes (L- selectin), endothelial cells (E- and P-selectins) and activated platelets (P-selectin) [17]. To achieve the stable interactions necessary for resistance to shear forces, selectins must interact with selectin ligands rapidly, with high tensile strength, and with quick dissociation rates [18,19]. These requirements are achieved due to binding of selectin with sialyl-Lewis^X containing glycoproteins.

L-selectin, is expressed by all myeloid cells, naïve T cells, and central memory T cells. P-selectin is constitutively expressed on lung endothelium and found on the surface of activated platelets and inflamed endothelial cells. E-selectin is expressed on inflamed endothelial cells and non-inflamed skin vasculature [6]. The respective ligands for these selectins are carbohydrate-containing structures that must be modified by specific glycosyltransferases in order to become fully functional [20]. Molecules such as PNAd (GlyCAM-1 and CD34) can interact with L-selectin and P-selectin to facilitate

tethering and rolling of naïve T cells and central memory T cells to lymph nodes. Alternatively, PSGL-1 and CLA can interact with P-selectin and E-selectin respectively to facilitate entry of memory and effector cells into inflamed organs [6] (Table 1.1).

Since selectin – selectin ligand interactions are temporally short, subsequent steps must occur in order for cells to firmly attach to endothelial cells; a process that requires the activation of integrins by chemokines.

Chemokines

Chemokines are small proteins that function to chemoattract cells by engaging G-protein coupled receptors (GPCRs) [21,22]. The chemokine family consists of over 40 proteins that are organized into subfamilies based on the position of cystein residues (CC, CXC, C, and CX_3C) [21]. The initiation of chemotaxis depends on the coupling of pertussis toxin sensitive Gαi proteins which orchestrate an inside-out signaling cascade that results in a number of outcomes (lymphoid tissue organization, antigen surveillance, and conformational changes to integrins) [23]. Once a leukocyte tethers and rolls along endothelium, the cell is brought into close contact with chemokines that are constitutively (homeostatic) or inducibly (inflammatory) expressed depending on the condition of the site in which the cell is trying to access [24,25]. Under physiological conditions, CCL21, CXCL13, and other chemokines are expressed on the surface of endothelial cells of HEVs which can bind to cognate receptors (i.e. CCR7 and CXCR5), located on naïve T cells, central memory T cells, B cells and other cell populations [21,26]. However, during inflammation, effector subsets of T cells can express high

levels of other chemokine receptors (CCR4, CCR8, CCR9, and CCR10) in order to access inflamed skin, lamina propriety or other sites via endothelial expression of CXCL12, CCL25 and other chemokines [21,25,27].

Chemokines play important roles not only in recruitment of cells to extravascular sites, but also in directing migration of leukocytes within those sites [21]. This is an important mechanism because it facilitates the organization of cells in secondary lymphoid organs in order to maintain homeostasis and initiate and direct adaptive immune responses [21]. However, to enter the lymph nodes, chemokines on the surface of high endothelial venues (i.e. CCL19) activate integrins so that firm adhesion to endothelium can be achieved.

Integrins

Integrins have been shown to facilitate tethering and rolling along endothelium to allow entry into organs in the absence of selectins. However, this process is not as efficient as selectin mediated tethering [28,29]. The most notable function of an integrin is its ability to firmly attach a mobile cell that is already rolling along the endothelium. The process of firm attachment is rapidly triggered by chemokine receptor activation such that the integrin can bind to a member of the immunoglobulin superfamily, ICAM1,2,3 or VCAM1, expressed on the surface of the endothelium [30,31]. Integrins are heterodimers consisting of an alpha chain and a beta chain and the integrins that contribute to leukocyte arrest typically belong to the β2-integrin (i.e. LFA-1), α4 integrins (i.e. VLA-4) and $\alpha 4\beta 7$ subfamilies (i.e. LPAM-1) [25,32]. The diversity of alpha and beta

chains contributes to specificity in integrin-mediated arrest such that chemokine triggered signaling might regulate distinct integrins and subsequent entry into specific locations [25]. However, there is some evidence against the idea of selective recruitment [33]. Nevertheless, this process of integrin activation could be due to expression levels of chemokine receptors or possibly the chemokine receptors affinity for a particular chemokine.

A migrating leukocyte typically expresses an inactivated integrin in a so called “bent-low-affinity” conformation. Upon activation of the integrin, the bent conformation changes to an “extended intermediate-affinity” or “high-affinity” conformation leading to the opening of its ligand-binding pocket, firm adhesion and extravasation [23].

Sequential Adhesion Cascade

The ability for leukocytes to leave the blood and enter extravascular compartments is dependent on the molecules that are expressed by the adherent leukocyte and the endothelial cell. The first step mediated by selectin-selectin ligand interactions facilitate the slowing down of the adherent cell through capture, tethering, and rolling. Once the leukocyte begins to resist the stress of shear forces, chemokines on the surface of the endothelium can provide stimulation to cognate chemokine receptors to induce an inside-out signal responsible for opening the ligand-binding pocket of specific integrins. The integrin can then interact with its respective cell adhesion molecule to facilitate firm arrest, allowing the cell to crawl along the surface of the endothelium and undergo extravasations. Each of these steps are required for

lymphocyte entry into secondary lymphoid organs (such as lymph nodes) and proper delivery of lymphocytes to inflamed sites. The ability for lymphocytes to express specific adhesion molecules along with specific chemokine receptors provide direction to those cells that enable them to be delivered to the correct address in the body in which they circulate.

T Cells

Hematopoietic stem cells in the bone marrow give rise to an array of progenitor populations. Majority of these hematopoietic cells mature in the bone marrow, but T cell development occurs in the thymus. The function of the thymus is to support a multistep process to yield mature, self-tolerant, and functional T cells [34,35]. Progenitor T cells produced in the bone marrow migrate towards the thymus via the blood and settle into the thymus likely through rolling, adhesion, and extravasations; a process similar to mature lymphocytes entering secondary lymphoid organs [36]. Once in the thymus, immature non-T cell receptor (TCR) bearing CD4/CD8 double negative (DN) subsets will undergo four developmental stages (DN1-DN4) based on the expression of CD44, CD117, and CD25 [37]. In the DN1 stage, T cells are defined as CD44+ CD117+ CD25- that demonstrate the ability to proliferate and generate both $\alpha\beta$ and $\gamma\delta$ T cell subsets [34]. Expression of CD25 on DN1 cells begins the DN2 (CD44+, CD117+,

CD25+) stage, which is also characterized by extensive proliferation. Over time, CD44 and CD117 are downregulated, which begins the DN3 stage. During the DN3 stage, the TCRβ, TCRγ, and TCRδ loci undergo recombination-activating gene (RAG) 1 and RAG2 mediated rearrangement and are subsequently checked for successful rearrangement [38]. Thymocytes containing a functional TCRβ chain can then associate with a pre-TCRα chain and a CD3 molecule to form the pre-TCR complex [34]. After the pre-TCR complex has been successfully checked and selected for αβ lineage differentiation, cells will downregulate CD25 to enter the DN4 stage. While in the DN4 stage, cells undergoing αβ lineage differentiation will expand and begin to express CD4 and CD8 coreceptors (double positive – DP) and begin the rearrangement of the TCRα locus. Conversely, γδ lineage differentiation is different from αβ differentiation. During the DN3 to DN4 stage, development of γδ cells does not require a pre-antigen receptor check; it only needs a single TCR-dependent event [39].

Once passing into the DP stage, RAG mediated TCRα chain rearrangement stops and the DP (CD4 and CD8) cells can undergo positive and negative selection. During positive selection, an intact three-dimensional microenvironment composed of cortical epithelial cells, dendritic cells, and macrophages, which express MHC-I and MHC-II is integral. DP thymocytes undergoing positive or negative selection will interact with self-major histocompatibility complex (MHC) and if the TCR-MHC affinity is strong the cell will likely undergo negative selection. It has been noted that TCR affinity for positive selection ligands is lower than TCR affinity for negative selection [40]. Majority of thymocytes undergoing selection succumb to self-tolerance and are

eliminated via apoptosis (~95%) [35]. However, the DP thymocytes that do survive positive selection will become single positive (SP) for CD4 or CD8. There are two models that explain how this process occurs. In the instructive model of lineage commitment, data suggests that whichever correceptor is engaged during the TCR-MHC interaction will provide a specific signal which guides the cell towards that specific lineage. Conversely, in the selective, or stochastic model, data suggests that CD4 or CD8 expression is turned off randomly, driving the thymocyte towards a specific lineage [41]. Once a thymocyte becomes CD4 or CD8 positive, sphingosine-1-phosphate receptor-1 (S1P1) expressed on its surface will regulate T-cell egress from the thymus. The S1P1 ligand, sphingosine-1-phosphate (S1P), is highly concentrated in the blood and will diffuse into the thymus, causing the thymocytes to follow the S1P chemotactic gradient [42]. Following the chemotactic gradient, thymocytes can exit the thymus and enter into the blood circulation where they have the ability to populate secondary lymphoid organs and recognize cognate antigen.

Characteristics of Naïve T cells

After successful development in the thymus, CD4 T cells will recognize antigen presented by self-MHC-II molecules, while CD8 T cells will recognize antigen presented by self-MHC-I molecules. This pool of mature cells, once in the blood, will begin to circulate through secondary lymphoid and non-lymphoid organs and have the capacity to display a strong reactivity to a wide spectrum of foreign antigens [43]. This capacity to recognize and respond to an antigen presented by a self-MHC molecule is quite

amazing given the fact that there is an extremely low frequency of T cells that are specific for any given antigen. However, T cells are able to overcome this obstacle because of their ability to continuously migrate to different specialized locations. This allows T cells to reach locations that give the T cells a greater opportunity to interact with cognate antigen [9] (Figure 1.3).

The primary locations where T cells become activated are secondary lymphoid organs [44]. The ability to enter into secondary lymphoid organs, such as lymph node, is due to the expression of L-selectin (CD62L) and CCR7 on the surface of the T cell along with the interactions of PNAd and CCL21 on the HEV [7]. Once in the lymph node, CCL19 and CCL21 chemokines direct T cells towards a T cell area [21]. While in the T cell area, naïve T cells have the capacity to interact with mature DCs that can display antigen so that an effector response can be initiated if cognate antigen is recognized [45].

Characteristics of Effector T cells

In the lymph node, if cognate antigen is recognized, naïve T cells become activated and proliferate. Upon activation, T cells begin to highly express CD44, downregulate L-selectin and CCR7, upregulate other adhesion-associated proteins (fucosyltransferase –IV and –VII), and transiently express CD69 which leads to transcriptional downregulation of S1P1 reducing the capacity for T cells to leave the LN [43]. Due to the downregulation of S1P1, effector T cells will be retained and as such accumulate in LNs where it is thought that the cells receive adequate programming

signals before exiting [46,47]. After about three days, effector T cells will become responsive to S1P and exit the lymph node using S1P1. Once in the efferent lymphatics, the T cells will enter the thoracic duct and subsequently end up in the blood circulation where the effector T cell can migrate towards the target organ [48,49,50]. Once at the target organ, CD8 T cells can exert cytotoxic effects and eventually most effector cells will die to give rise to a stable memory pool [51].

Two Classes of Memory T cells

Memory T cells have been classified as central memory (Tcm) or effector memory (Tem) based on the expression of CD44, L-selectin, CCR7, and differences in homing. Memory T cells that are $CD44^{high}$, $CD62L^{high}$ and CCR7+ have been classified as central memory T cells, while those that are $CD44^{high}$, $CD62L^{low}$ and CCR7- are classified as effector memory T cells [52]. When stimulated, effector memory T cells exhibit effector cytokines more rapidly and proliferate faster than their central memory counterparts [52]. Central memory T cells preferentially home to secondary lymphoid organs while effector memory T cells are preferentially located in peripheral sites [5] (Figure 1.4).

LCMV Response

Lymphocytic choriomeningitis virus (LCMV) is an arenavirus and a natural mouse pathogen [53]. The virus, which has multiple strains (both acute and chronic), is a well-

established viral model for studying CD8 T cell mediated responses [54]. During acute LCMV (Armstrong strain) infection, a robust CD8 T cell response is elicited in spleen and lymph node and is thought to be controlled primarily by CD8 T cells. However, CD4 and antibody specific B cells are also generated in the response [55,56]. In the case of chronic LCMV infection (Clone 13 strain), the high antigen load causes clonal exhaustion of CD8 T cells, leading to persistent infection [57]. In this thesis, we exclusively use the acute infection of LCMV (Armstrong strain) in order to assess how inflammatory conditions affect T cell trafficking.

T cell Trafficking

It is widely held that naïve T cells continuously circulate between secondary lymphoid organs and the blood and that activated and memory T cells migrate through non-lymphoid organs [7]. The data for this belief originated from an early study performed in sheep where memory T cells were found in afferent lymph draining towards the lymph node and naïve T cells were found in the efferent lymph draining away from the lymph node [5]. Considering the fact that non-lymphoid organs do not have HEVs and that naïve T cells primarily use HEVs to enter secondary lymphoid organs, it led to the idea that these cells possessed no mechanisms by which to enter non-lymphoid organs. Contrary to this, data has existed in the literature to suggest that naïve T cells have the capacity to enter non-lymphoid organs [58,59]. In one study, it was found that 30 minutes after intravenous injection of radiolabeled T cells into rats, about half of the injected T cells entered secondary lymphoid organs while the other half

entered non-lymphoid organs [58]. The observation that naïve T cells can enter non-lymphoid organs suggests that there are numerous molecular adhesion mechanisms that facilitate transmigration and that no one subset of cells is preferred. However, what is clear is that certain adhesion molecules must undergo post-translational modifications in order to become fully functional.

Glycosylation in Immunity

Covering the surface of nearly all mammalian cells are oligosaccharide-rich post-translationally modified proteins that are attached to other membrane bound proteins and lipids [20]. This layer of proteins is termed the glycocalyx and is a property of cells that allows the cell to interact with its environment. The glycocalyx consists of asparagine-linked glycans (N-linked), serine/threonine linked glycans (O-linked) and lipid-linked glycans (glycolipids) [20]. The glycoconjugates represent linear and branched polymers of a limited monosaccharide diversity linked through glycosidic bonds [20]. However, despite the limited number of monosaccharides, the combinatorial arrangement of the monosaccharides make these structures extremely diverse. Assembly of the glycoconjugates occurs through the use of glycosyltransferases, which are type II transmembrane proteins that contain their catalytic domain in the lumen of the Golgi apparatus. Every glycosyltransferase is classified according to the monosaccharide it requires and also by the way it constructs

a glycosidic linkage (alpha-anomeric or beta-anomeric). The manner in which the linkage is formed is indicated by numbers that denote which carbon atoms are involved in the glycosidic bond between the monosaccharide and acceptor substrate [20]. Two glycosyltransferases that have been implicated in the formation of selectin ligands are fucosyltransferases–IV and –VII [60,61,62,63,64]. Fucosyltransferases –IV and –VII catalyze the formation of an alpha anomeric glycosidic bond between carbon 1 on fucose and carbon 3 of the acceptor substrate (GlcNAc) (Figure 1.2).

Fucosyltransferase-IV and Fucosyltransferase-VII

Considerable research has been conducted assessing the importance of alpha1,3-fucosyltransferases –IV and –VII in immune cell trafficking (Table 1.3). In 1996, Lowe conducted a study looking at the phenotype of mice that lacked a targeted disruption of the fucosyltransferase-VII gene [64]. They observed blood leukocytosis and deficits in the expression of L-, E-, and P-selectin ligands as well as decreased neutrophil, Th1, and Tc1 trafficking in inflammation using a contact hypersensitivity model [20]. Subsequent experiments looking into the role of fucosyltransferase-IV have shown that this enzyme plays an independent role in selectin-dependent recruitment of leukocytes and that it contributes to HEV-derived L-selectin ligands, but that fucosyltransferase-VII has a more prominant role [65]. Additional experiments looking at the absence of both fucosyltransferases –IV and –VII has elucidated the importance of these glycosyltransferases as lymphocyte homing to lymph node is severely reduced in their absence [62]. The interesting feature of this significant defect is that despite the

enzymes absence, CD4+ and CD8+ T cells are still able to mount a response to the LCMV model of infection in visceral organs by becoming primed in the spleen [60]. This data was further corroborated in the *M. tuberculosis* model of infection where the data showed similar granuloma formation compared to WT [63]. These data suggested that despite reduced total cellularity in the lymph nodes, a response could be initiated using selectin ligand independent mechanisms. However, in the WT mouse fucosyltransferase –IV and –VII are not the only enzymes that play a role in L-selectin–selectin ligand-binding activity. An additional enzyme modification by a sulfotransferase is required.

Sulfotransferases

Other integral glycosyltransferases in the formation of L-selectin ligands are the GlcNAc-6-O-sulfotransferases [66]. The two enzymes in this group, Glcnac6st1 and Glcnac6st2, function to add a sulfate group to the N-acetylglucosamine (GlcNAc) on O-glycans [67]. Rosen and colleagues found that the presence of a fucose residue in the sialyl-Lewis^{X} moiety was not sufficient to mediate lymphocyte tethering and rolling using L-selectin-selectin ligand interaction [68]. Since L-selectin is the primary selectin used by naïve T lymphocytes and central memory T cells to tether and roll along high endothelial venules at LNs, the proper modification of L-selectin ligands is critical for proper trafficking into LN.

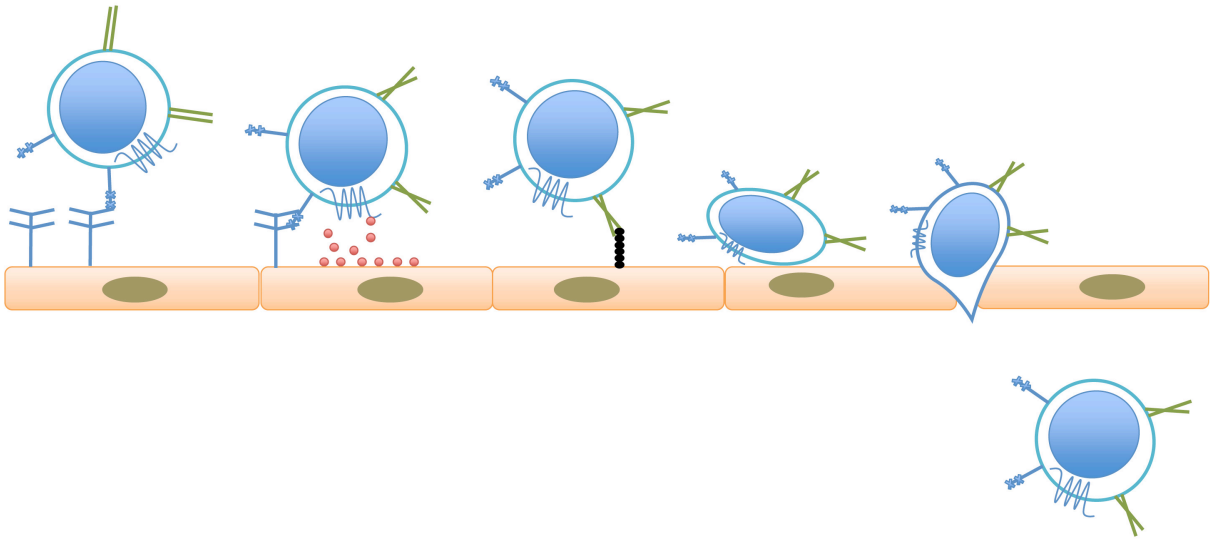
In order for increased L-selectin-selectin ligand interactions to occur, sulfation of GlcNAc at the 6th carbon position to form 6-sulfo sialyl-Lewis^{X} needed to occur to

enhance binding. Using knockout mice for different GlcNAc-6-O-sulfotransferases (Glcnac6st1 -/-, Glcnac6st2 -/-, and Glcnac6st1-/- Glcnac6st2 -/- DKO) showed that the absence of the enzymes contributed to a number of different phenotypes. Glcnac6st1-/- Glcnac6st2 -/- DKO mice demonstrated an abolishment of rolling on HEVs [67,69]. However Glcnac6st1 -/- [70]and Glcnac6st2 -/- [71] single knockouts demonstrated an overlapping function with Glcnac6st2 -/- mice revealing higher rolling velocities on HEVs compared to Glcnac6st1 -/- mice. These experiments demonstrate that contribution to selectin-selectin ligand specificity is complex and that there are a number of different players that contribute to and are necessary for successful entry into lymph nodes.

Experimental Objectives

In the following studies presented in this thesis, we explore the role of fucosyltransferases –IV and –VII in T cell trafficking. Specifically we test the following hypotheses: 1) Naïve T cells accumulate in non-lymphoid organs when entry to LNs is impaired; 2) FtDKO mice show changes in the lung environment, which allow selective migration of T cells to that site; 3) Memory T cells use selectin ligand independent mechanisms to enter lymph nodes; and 4) Memory T cells are selectively retained in lymph nodes of FtDKO mice. We present evidence that support the hypotheses that naïve T cells accumulate in non-lymphoid organs, like the lung, when entry to LN is impaired and that memory T cells show increased homeostatic expansion and longer residence in LNs with low T cell density.

Appendix



Tethering and Rolling Selectin Mediated	Activation Chemokine Mediated	Firm Attachment Integrin Mediated	Psuedopodia Formation	Extravasation	Tissue Migration

Figure 1.1. Mechanisms of T cell migration. Naïve T cell or central memory T cells migrate across HEVs into lymph node using L-selectin, CCR7, and LFA1. Initial tethering and rolling is mediated by selectin-selectin ligand interactions, followed by the binding of chemokines to chemokine receptors, and then firm attachment via integrins. Firm attachment will induce pseudopodia formation and subsequent extravasation into organs.

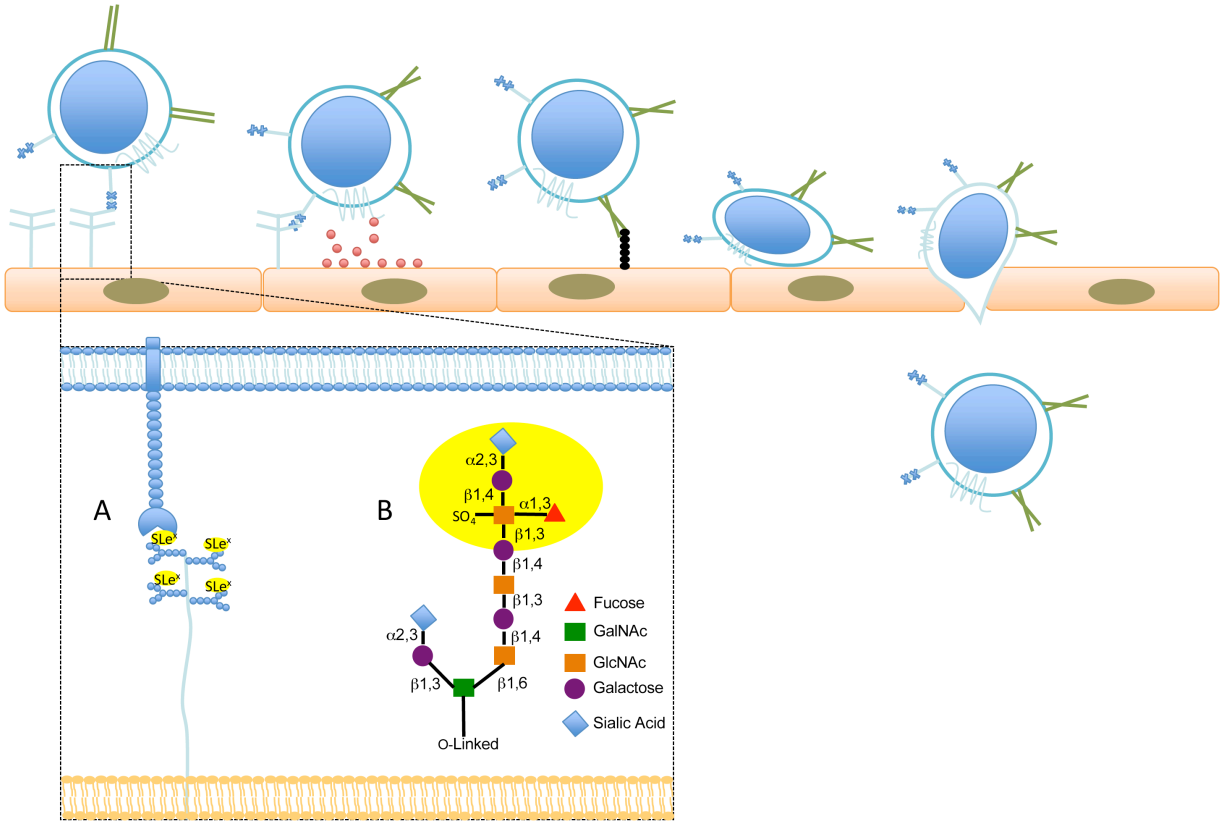


Figure 1.2. Depiction of selectin – selectin ligand interaction. A) L-selectin from an adherent cell interacts with its HEV bound sialyl-Lewis^X ligand on PNAd. B) α1,3 fucosyltransferases catalyze the formation of an alpha anomeric glycosidic bond between carbon 1 of fucose and carbon 3 of the GlcNAc acceptor. Additional sulfation by GlcNAc-6-O-sulfotransferases forms the 6-suflo sialyl-Lewis^X moieties that are critical for selectin ligand mediated tethering and rolling along the endothelium.

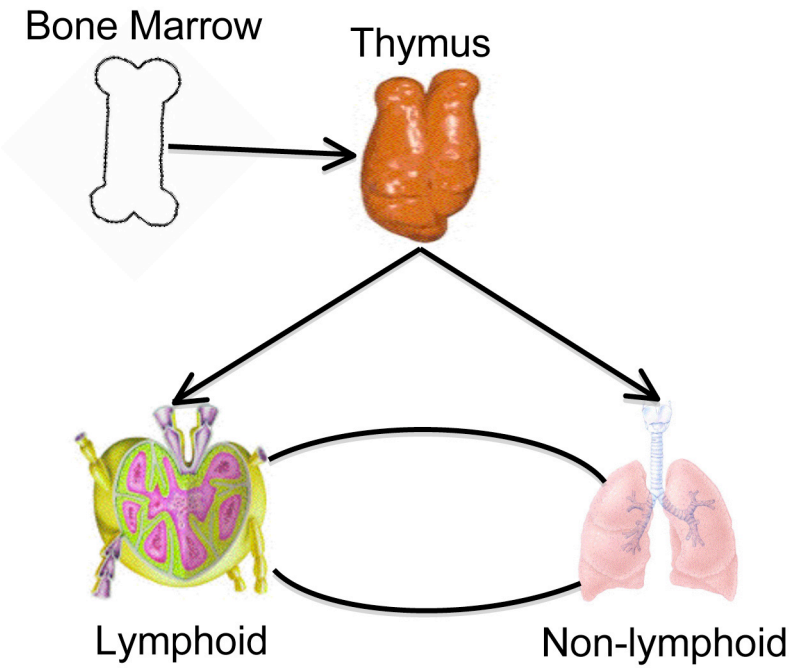


Figure 1.3. Naïve T cell trafficking. T cell precursers migrate from the bone marrow and develop in the thymus where they undergo negative and positive selection. Mature T cells then leave the thymus and migrate through the blood and circulate through secondary lymphoid organs as well as non-lymphoid organs. Naïve T cells will continue to re-circulate until they come in contact with cognate antigen or die.

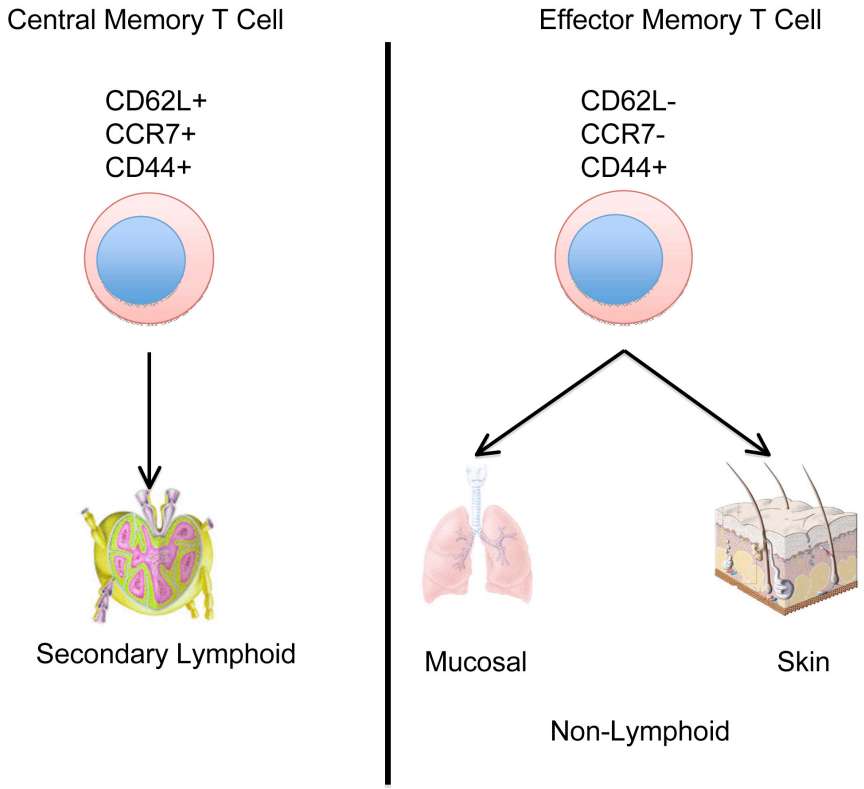


Figure 1.4. Memory T cell trafficking. Central memory T cells express L-selectin and CCR7 that allow them entry to LNs via HEVs. These cells primarily reside in secondary lymphoid organs and rapidly proliferate following Ag exposure. Effector memory T cells do not express L-selectin and primarily reside in non-lymphoid organs. Upon activation, effector memory T cells rapidly elicit effector functions such as section of effector cytokines (i.e. IFNg and Granzyme B).

Table 1.1. Expression of Selectins and selectin ligands. (Adapted from von Andrian and Mackay 2000)

Selectins and Selectin Ligands		
Adhesion Molecule	**Distribution**	**Regulation of Function and Expression**
Selectins		
L-Selectin	All leukocytes except effector and effector memory T cells	Rapidly shed upon activation
E-Selectin	Endothelial cells	Expression induced by TNF-_ and IL-1
P-selectin	Endothelial cells and platelets	Stored Intracellulary in resting cells. Rapidly expressed upon activation
Selectin Ligand		
Sialyl-Lewis^X	Activated T cells and HEVs	Expression depends on fucosyltransferase-IV and -VII

Table 1.2. Glycoprotein substrates that contain the Sialyl-Lewis^{X} structure. (Adapted from von Andrian and Mackay 2000)

Glycoprotein substrates that contain the Sialyl-Lewis^{X} structure	
P-selectin glycoprotein ligand 1 (PSGL-1)	Leukocytes
Peripheral-node addressin (PNAd)	HEVs and at sites of chronic inflammation
Cutaneous lymphocyte antigen (CLA)	Skin-homing T cells, dendritic cells, and granulocytes

Table 1.3. Glycosyltransferases relevant to selectin ligand biosynthesis. (Adapted from Ley and Kansas 2004)

Enzyme	Involved in biosynthesis of ligand for	Expression
Fucosyltransferase-VII	L-, E-, and P-selectin	Activated T cells and HEVs
Fucosyltransferase-IV	L-, E-, and P-selectin	HEVs
Core 2 β1,6-glucosaminyltransferase	L- and P-selectin	Myeloid cells, activated T and B cells
Sialyl 3-galactosyltransferase	L-, E-, and P-selectin	Ubiquitous
β1,4-galactosyltransferase	P-selectin	Ubiquitous
Tyrosine protein sulphotransferase 1,2	L- and P-selectin	Ubiquitous
HEC-glucosaminylsulphotransferase 1,2	L-selectin	HEVs and chronically inflamed endothelial cells

Chapter 2

Naïve T cells re-distribute to the lungs of selectin ligand deficient mice

Research described in this chapter is a modified version of an article published in 2010 in PLoS One by John R. Harp and Thandi M. Onami

Harp J.R., T.M. Onami (2010) Naïve T cells redistribute to the lungs of selectin ligand deficient mice. *PLoS One* 5: e10973

In this chapter, “our” and “we” refer to Thandi Onami and myself. My contribution to this paper includes: 1) planning experiments, 2) analyzing data, 3) preparing figures, and 4) editing.

Abstract

Selectin mediated tethering represents one of the earliest steps in T cell extravasation into lymph nodes via high endothelial venules and is dependent on the biosynthesis of sialyl Lewis X (sLe^x) ligands by several glycosyltransferases, including two fucosyltransferases, fucosyltransferase-IV and –VII. Selectin mediated binding also plays a key role in T cell entry to inflamed organs. To understand how loss of selectin ligands (sLe^x) influences T cell migration to the lung, we examined fucosyltransferase-IV and –VII double knockout (FtDKO) mice. We discovered that FtDKO mice showed significant increases (~5-fold) in numbers of naïve T cells in non-inflamed lung parenchyma with no evidence of induced bronchus-associated lymphoid tissue. In contrast, activated T cells were reduced in inflamed lungs of FtDKO mice following viral infection, consistent with the established role of selectin mediated T cell extravasation into inflamed lung. Adoptive transfer of T cells into FtDKO mice revealed impaired T cell entry to lymph nodes, but selective accumulation in non-lymphoid organs. Moreover,

inhibition of T cell entry to the lymph nodes by blockade of L-selectin, or treatment of T cells with pertussis toxin to inhibit chemokine dependent G-coupled receptor signaling, also resulted in increased T cells in non-lymphoid organs. Conversely, inhibition of T cell egress from lymph nodes using FTY720 agonisim of S1P1 impaired T cell migration into non-lymphoid organs. Taken together, our results suggest that impaired T cell entry into lymph nodes via high endothelial venules due to genetic deficiency of selectin ligands results in the selective re-distribution and accumulation of T cells in non-lymphoid organs, and correlates with their increased frequency in the blood. Re-distribution of T cells into organs could potentially play a role in the initiation of T cell mediated organ diseases.

Introduction

Lymphocytes are highly migratory cells. Uncovering pathways that allow these cells to access tissues not only facilitates our understanding of cell migration, but deepens our understanding of the relationship between immunity and immunopathology. Blood borne leukocytes, such as T lymphocytes, are recruited to extra-vascular sites, and these processes are highly regulated so that distinct cell populations are delivered to intended site(s) in the proper physiological or pathological context (reviewed in[20,72,73]). Imperfections in these processes may lead to qualitatively or quantitatively inappropriate delivery of cells to tissue sites that may result

in inflammatory pathological processes (reviewed in [20]). Several leukocyte adhesion deficiency (LAD) diseases in humans have been described, including LAD II, a congenital immunodeficiency disease characterized by patients having defective production of fucosylated ligands necessary for selectin mediated tethering[74,75,76,77].

Selectins (E-selectin, P-selectin, and L-selectin) are type I transmembrane glycan binding proteins (GBPs), or C-type lectins, that regulate leukocyte-endothelial cell adhesive interactions, critically involved in T lymphocyte trafficking from the blood to extra-vascular compartments [20]. Selectins interact selectively with sialylated fucosylated lactosamine structures (sialyl Lewis x or sLe^x) of membrane-associated mucin type glycoproteins such as GlyCAM-1, CD34, and MAdCAM-1 via binding by amino terminal carbohydrate recognition domains (CRDs). The a1,3 fucosyltransferases catalyze the synthesis of the sLe^x moiety, and thus these enzymes are essential in the elaboration of selectin ligands, although additional modification by sulfotransferases contributes to L-selectin binding [6,64,67,78]. Naïve and central memory T lymphocytes express leukocyte selectin, or L-selectin, while activated T lymphocytes downregulate L-selectin, but begin to express E- and P- selectin ligands as a consequence of inducible expression of a1,3 fucosyltransferases [79,80,81]. High endothelial cells which line functionally distinct high endothelial venules (HEVs) in different lymph nodes, constitutively express a1,3 fucosyltransferases, and this allows shear-dependent adhesive interactions between lymphocytes and selectin ligands on HEVs [62,82]. Following initial selectin-mediated tethering of T cells, chemokine receptor binding of

chemokines, such as CCR7 binding CCL21, activates G-coupled receptor signaling, which induces a conformational change of T cell integrins, such as LFA-1 [83]. Binding of high affinity LFA-1 on T cells leads to firm arrest and facilitates transendothelial migration into the lymph node (LN) [83,84].

Mice lacking the a1,3 fucosyltransferases were generated several years ago and demonstrate loss of selectin binding activity, impaired T cell migration to draining lymph nodes but normal migration to spleen, as well as impaired T cell extravasation to skin in both contact hypersensitivity and viral models [60,79]. However, knockout mice of one or both enzymes are able to mount potent immune responses as evidenced by clearance of lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and vaccinia viral infection in brain and ovaries, despite reduction of lymph node T cell numbers [60,61,79]. Moreover, examination of fucosyltransferase-VII and-IV double knockout mice (FtDKO) for immunity following infection with *Mycobacterium tuberculosis* found that FtDKO mice had significantly diminished numbers of activated CD8 and CD4 T cells in the draining lymph node of the lung (MedLN), but had effective granuloma responses and similar recruitment of activated T cells into the lung at later stages of infection [63]. Importantly, *Mycobacterium tuberculosis* infection of FtDKO mice demonstrated that iBALT develops similarly in the lungs of FtDKO mice, and express CXCL13, CCL21, and CCL19 chemokines [63]. This reduction in T cells in the MedLN of FtDKO mice, even under conditions of chronic inflammation by an intracellular pathogen, convincingly suggests that no major alternative mechanism of cell recruitment compensates for the deficiency of a1,3 fucosyltransferases in T cell

homing to the LN [63].

We were interested in determining how loss of selectin ligands affects immunity at mucosal sites [85]. Here we report our discovery that FtDKO mice display a significant increase in naïve T cell populations located in the lung. This phenotype has never been reported in these mice, so we further explored the contribution of selectin ligands to T cell migration into non-lymphoid organs. We examined T cell trafficking in FtDKO mice under non-inflammatory conditions, as well as following inflammation due to viral infection. The data presented here suggests that in FtDKO mice, T cells selectively migrate and accumulate in non-lymphoid organs under non-inflammatory conditions. However, under inflammatory conditions, T cell trafficking of Ag-specific effector and memory T cell populations to non-lymphoid organs was reduced in FtDKO mice, consistent with previous studies [86,87,88]. Finally, to investigate why naïve T cells would preferentially accumulate in non-lymphoid organs of FtDKO mice under non-inflammatory conditions, we considered two explanations: 1) a specific increase in trafficking due to loss of selectin ligand expression in the lungs of FtDKO, making them more permissive to T cell entry, or 2) a non-specific increase in trafficking to non-lymphoid organs due to T cell re-distribution when cells cannot enter LNs. We tested the latter hypothesis and our results are consistent with this hypothesis. Treatments that block T cell entry into the LN result in selectively increased numbers of T cells in non-lymphoid organs. Conversely, treatments that blocked T cell exit from the LN showed reduced T cell trafficking to non-lymphoid organs. Thus, under conditions where T cell entry to LNs is impaired, T cells may accumulate in the perivascular or interstitial

campartments of the lung, and this altered distribution could possibly play a role in the initiation of lung diseases.

Results

Increased numbers of naïve T cells in the lungs of FtDKO mice

We isolated lymphocytes from organs of naïve WT and FtDKO mice and analyzed CD4 and CD8 T populations at each site. As expected, CD4 and CD8 T cell populations were significantly reduced (~70%) in mediastinal lymph node (MedLN) (Figure 2.1A, C). CD8 T cell populations were also reduced in the O-NALT. Surprisingly, we observed ~5-fold increase in T cell numbers in the lungs of FtDKO mice, and we also consistently observed a modest increase in T cell numbers in the spleen (~20% increase) (Figure 2.1A, C). B cell populations were also increased in the lung, about 2-fold (data not shown). Phenotypic analysis of T cell populations in organs of the FtDKO mice reveal that similar to WT mice, the majority of T cells in the spleen and lung were naïve ($CD44^{lo}$ and $CD62L^{high}$) (Figure 2.1B and data not shown). However, in the MedLN and O-NALT of FtDKO mice, there was a significant decrease in naïve ($CD44^{lo}$) T cell populations (Figure 2.1B, D).

Since we saw increased numbers of T cells in the lungs of FtDKO mice, we hypothesized that similar to CCR7-/- mice, FtDKO mice may show evidence of neo-

lymphoid follicles or induced bronchus-associated lymphoid tissue (iBALT) in the lungs [89]. However, H&E analysis of lung sections from FtDKO showed no evidence of iBALT in any lung sections examined (Figure 2.1E). Analysis of T cells in lung sections suggested increased T cells in the perivascular compartment, consistent with the observed increase in T cell numbers by FACS analysis (Figure 2.1F). Taken together, these observations raised the possibility that naïve T cells in FtDKO mice were re-distributing to other organs, including non-lymphoid organs such as the lung.

Migration of Ag-specific CD8 T cells to inflamed lung is impaired in FtDKO mice

The role of selectin mediated recruitment of leukocytes to inflamed tissues is well established [86,87,90]. Following inflammatory insult, E- and P-selectin are upregulated on inflamed endothelial cells allowing for the recruitment of selectin ligand expressing leukocytes. Naïve T cells express L-selectin, but not selectin ligands, while activated T cells downregulate L-selectin, both transcriptionally and via proteolytic cleavage, and induce expression of a1,3 fucosyltransferases, resulting in selectin ligand expression[81,91,92,93].

We examined the expansion of Ag-specific effector and memory CD8 T cell populations in the lungs of WT and FtDKO mice following LCMV Armstrong infection. At day 8 and day 60 post-infection, we observed significant expansion of viral specific CD8 T cells in organs of FtDKO mice (Figure 2.2A, B). However, CD8 T cell numbers were significantly reduced (~50%) in the lungs of FtDKO mice compared to WT mice,

consistent with the described role of selectin-mediated homing to inflamed lung (Figure 2.2C, D). Similar to a recent study, CD8 T cell numbers in the MedLN following infection remained severely reduced (~85%) (Figure 2.2 C, D) [63]. Interestingly, we noted that at day 60, Ag-specific memory CD8 T cells in MedLN and O-NALT were not reduced (Figure 2.2B, D). In fact, FtDKO LN showed significant enrichment of Ag-specific memory CD8 T cells compared to the total CD8 population (Figure 2.2 and J. R. Harp and T. M. Onami manuscript in preparation).

Naïve and memory T cells preferentially accumulate in non-lymphoid organs in FtDKO mice

Paradoxically, our results following infection show that effector and memory Ag-specific CD8 T cell populations were reduced in the inflamed lungs of FtDKO mice, while naïve CD8 populations were increased in the lungs of uninfected FtDKO mice (Figures 2.1 and 2.2). Previously published studies have shown that defective T cell migration to inflamed lungs was due to loss of expression of selectin ligands by T cells—i.e. defective migration to inflamed tissues in FtDKO mice is T cell intrinsic [88]. However, defective T cell migration to LNs is due to loss of expression of selectin ligands on HEVs [62,88]. In FtDKO mice, selectin ligand expression is absent in all cells, including activated T cells.

To determine whether naïve or memory CD8 T cells, capable of expressing selectin ligands, selectively homed to the lung of FtDKO mice, we performed adoptive

transfer of *bona fide* P14.Tg WT naïve and central memory CD8 T cells into WT or FtDKO recipient mice under non-inflammatory conditions. As expected, transferred T cells were significantly impaired in homing to LNs as well as O-NALT in FtDKO mice (Figure 2.3B). In contrast, we observed that transferred naïve and memory CD8 T cells show increased numbers in the spleen, lung, and liver of FtDKO mice compared to WT mice (Figure 2.3B, C). We saw no evidence of transferred T cells in the bronchial alveolar lavage (BAL) (data not shown). The majority of transferred CD8 T cell populations were located in the spleens of both WT and FtDKO mice (79% and 82% respectively). The remaining CD8 T cells show differences in their distribution comparing blood, liver, lung, cervical lymph node (CLN), iliac lymph node (ILN), and MedLN (Figure 2.3C). In WT recipient mice, the majority of the remaining transferred CD8 T cells preferentially homed to the LNs with a small fraction in non-lymphoid organs (Figure 2.3C). In contrast, in FtDKO mice, the majority of the remaining transferred CD8 T cells were preferentially located in the lung and liver, with few entering the LNs (Figure 2.3C). Thus, consistent with our observation of increased numbers of naïve T cells in non-lymphoid organs of FtDKO mice, these data demonstrated that naïve and central memory CD8 T cells preferentially accumulated in non-lymphoid organs in FtDKO mice.

Inhibition of naïve T cell trafficking to LN by Pertussis toxin treatment results in selective re-distribution to non-lymphoid organs

The observation of increased numbers of T cells in the lungs of FtDKO mice could be due to several possibilities. One possibility is that loss of selectin ligand expression in the FtDKO mice results in a change in the lung environment, making the lung more permissive for T cell entry. A recent report observed that reduced sLe^x expression on lung epithelial cells contributes to defects in airway wound repair [94]. Additionally, defects in core fucosylation have been associated with abnormal lung development and an emphysema-like phenotype in Fut8 deficient mice [95,96]. Another possibility is that by blocking T cell entry to the LN, T cells are re-distributed to other sites. One place these naïve T cells end up is non-lymphoid organs, if these sites are part of the normal migratory pathway of naïve T cells [9,12,97]. To test this hypothesis, we examined whether inhibition of chemokine receptor signaling by pertussis toxin treatment also results in increased migration of naïve CD8 T cells to lung and other non-lymphoid organs of recipient mice. In Figure 2.4, we show that as expected, Ptx treatment of naïve CD8 T cells resulted in significantly impaired trafficking of transferred cells to LNs, observed as decreased percentages and overall numbers of transferred Thy1.1 cells in CLN, ILN, and MedLN (Figure 2.4B, C). In contrast, significantly more naïve CD8 T cells were observed in the lung and liver (Figure 2.4C). We observed no differences in numbers of transferred naïve CD8 T cells in spleen and bone marrow. Notably, we observed an increased concentration of transferred Ptx treated T cells in

the blood (Figure 2.4C). Additionally, if we treated recipient mice with blocking antibodies to L-selectin (anti-CD62L), we also observed reductions in naïve CD8 T cells in LNs, and increased T cells in lung and liver (Figure 2.6A and data not shown). Taken together, these data support our hypothesis that general inhibition of T cell trafficking to LN results in increased T cell re-distribution to non-lymphoid organs.

Retention of T cells in LN by FTY720 treatment results in reduced naïve CD8 T cell populations in non-lymphoid organs

Thus far, our experiments demonstrated that when we inhibited entry of naïve CD8 T cells via HEVs to LNs, we observed increases in the number of T cells in lung and liver (Figure 2.3-2.4). Based on this result, we predicted that if we blocked T cell egress from the LN, we would observe reduced T cells in the lung and liver. To test this hypothesis, we transferred naïve CD8 T cells into recipient mice, and treated these mice with FTY720, an agonist for S1P1, which inhibits T cell egress from the LN [98,99]. Treatment with FTY720 generally resulted in increased total lymphocytes in LNs and a significant drop in total lymphocytes in the PBMC (Figure 2.5C). FACS analysis and enumeration of the transferred naïve CD8 T cell population revealed that transferred cells in treated mice were significantly lower in the liver, lung, and PBMC while remaining unchanged in other organs (Figure 2.5D). Our study using *bone fide* naïve T cells supports and extends data in a recent paper showing FTY720 treatment depletes endogenous naïve phenotype T cells in peripheral organs [99].

Relationship of naïve T cells in non-lymphoid organs and T cells in blood

If we focused our analysis on naïve CD8 T cells, a clear inverse relationship emerges from our data (Figure 2.6). We used several methods to block T cell entry to LN, including genetic ablation of selectin ligands (FtDKO mice), inhibition of G-coupled dependent chemokine receptor signaling in T cells, or anti-CD62L treatment of mice to inhibit CD62L dependent migration (Figure 2.6). In general, under conditions of impaired migration to LN, we observed increased migration to non-lymphoid compartments such as lung and liver (Figure 2.6A). In contrast, S1P1 agonism by FTY720 treatment of mice results in retention of transferred naïve CD8 T cells in LN, but resulted in a drop in naïve T cells in non-lymphoid organs (Figure 2.6A). Taken together, the proportion of naïve T cells located in non-lymphoid organs was inversely correlated with the proportion of naïve T cells in the LN (Figure 2.6A).

Under normal conditions naïve T cells emerge from the thymus and migrate via the circulation into secondary lymphoid organs and exit LNs via the lymphatic vessels, culminating at the thoracic duct, which drains into the superior vena cava, returning T cells to the blood. We reasoned that treatments that inhibit naïve T cells in the blood from entering the LN via HEVs, would result in higher proportions of naïve T cells remaining in the blood. This higher proportion of T cells in the blood could result in higher numbers of T cells that transit into the lung via the lung's dual blood supplies, the pulmonary and bronchial arteries, where T cells could accumulate in the lung perivascular and interstitial compartments [12,100]. However, in the mouse it is

generally agreed that there is limited blood supply from the bronchial arteries, so the pulmonary arteries are the only blood supply to the lung, and T cells may enter lung compartments after being sequestered in the pulmonary capillary bed [101,102,103]. Conversely, treatments that retain T cells in the LN, could reduce the proportion of T cells in the blood, limiting the number of T cells that may potentially enter the lung.

We analyzed the proportion of transferred naïve CD8 T cells in the blood following inhibition of LN entry, or inhibition of LN exit. For each treatment, we calculated the ratio of the concentration of naïve T cells in the blood in treated versus untreated groups of mice. We then considered whether this resulted in more T cells in LN or non-lymphoid organs. Schematically, we show that there is an inverse relationship between the concentration of naïve T cells in the blood and localization in LN versus non-lymphoid organs (Figure 2.6B). Generally, when we inhibited naïve CD8 T cell entry to LN, via the HEVs using CD62L antibody blockade, genetic deficiency of selectin ligands, or inhibition of chemokine receptor signaling via G-coupled receptor signaling pathways, we observed more naïve CD8 T cells in the blood, and we also observed more naïve CD8 T cells in non-lymphoid organs (Figure 2.6B). Conversely, when we inhibited naïve CD8 T cell exit from the LN, via S1P1 agonism, fewer naïve CD8 T cells were observed in the blood, and we observed fewer naïve CD8 T cells in non-lymphoid organs (Figure 2.6B). Under homeostatic conditions, the majority of naïve T cells preferentially home from the blood via HEVs into the LN, and we observe a small, but measurable population of naïve T cells in non-lymphoid organs (Figure 2.1 and 2.6). Thus, our observation of increased naïve T cells in the lungs of FtDKO mice is

consistent with our hypothesis that impaired T cell migration to secondary lymphoid organs such as LN, results in increased T cells in the blood, and subsequently results in migration and preferential accumulation in non-lymphoid organs.

Discussion

The focal event of the adaptive immune response is the encounter between naïve T cells and APCs displaying specific antigen, which results in T cell activation. Thus, our understanding of the immune system will be critically dependent on characterizing the molecular events that regulate and guide highly migratory T cells and APCs to appropriate microenvironments [104]. In this study, we have discovered a previously unknown phenotype in FtDKO mice. We observed increased numbers of naïve T cells in the lungs with no evidence of iBALT formation, and we demonstrated that T cells selectively re-distribute to the lung, as well as several other organs in FtDKO mice (Figure 2.1, 2.3). Viral infection of FtDKO mice to induce inflammation and trigger viral-specific T cell expansion, demonstrated that while there was robust CD8 T cell expansion and viral clearance by day 8, Ag-specific effector and memory CD8 T cell populations were significantly reduced in the lungs of FtDKO mice, consistent with the described role of selectin ligands in T cell extravasation to inflamed organs (Figure 2.2 and data not shown). Taken together, our data suggests that both selectin ligand-dependent and selectin ligand-independent mechanisms contribute to T cell migration to non-lymphoid organs under inflammatory and non-inflammatory conditions,

respectively.

To further understand the mechanism of T cell re-distribution and accumulation in non-lymphoid organs under non-inflammatory conditions, we transferred *bona fide* naïve and memory CD8 T cells and enumerated them in organs. However, it must be considered that T cell migration is a very dynamic process and our analysis was limited to one time point (18hrs post-transfer). Earlier or later times may have shown differences in organ distribution of these transferred T cells. Nonetheless, our results demonstrated preferential accumulation of both naïve and central memory T cell populations in non-lymphoid organs of FtDKO mice (Figure 2.3). This observation is in contrast to the main dogma that suggests these cells do not enter non-lymphoid organs, but fits well with alternatively proposed migration models that suggest T cells continuously enter lymphoid and non-lymphoid organs [24,33,105]. Moreover, we found that conditions that inhibit T cell trafficking to the LN, and increase the proportion of T cells in the blood, such as Ptx treatment and anti-CD62L blockade, also result in increased naïve CD8 T cell trafficking to non-lymphoid organs (Figure 2.4 and 2.6). In contrast, inhibition of T cell exit from the LN by FTY720 agonism of S1P1, which drops the proportion of T cells in the blood, significantly reduced the number of naïve CD8 T cells in lung and liver (Figure 2.5 and 2.6). Similarly, transfer of naïve CD4 T cells into anti-CD62L treated recipient mice or FTY720 treated mice also showed alterations of CD4 T cell numbers in the lung (S. Caucheteux and W.E. Paul, unpublished observation). Taken together, these data suggest that when T cell trafficking to LN is altered, this influences the concentration of T cells in the blood that enter the spleen and

non-lymphoid organs such as the lung and liver. While this does not preclude the possibility that additional changes in peripheral tissues of FtDKO mice may also influence trafficking of T cells, it does suggest that altered T cell trafficking to LNs is sufficient to cause increased T cell accumulation in several organs.

Normal re-circulation of T cells to secondary lymphoid organs such as LN, spleen, and mucosa associated lymphoid tissues such as O-NALT, is thought to be important for T cell interaction with stromal cells expressing IL-7 and other survival cytokines [106,107]. As noted above, there is controversy in the literature regarding whether naïve T cells normally migrate into non-lymphoid organs. Several reviews and textbook discussions report that only effector and memory T cells enter non-lymphoid organs, and naïve T cells do not normally enter these sites [5,6,24]. However, other studies have reported that as part of the normal migratory pathway, naïve T cells enter non-lymphoid organs including lung, but naïve T cell residency time in these organs is short compared to effector/memory T cells [9,12,13,33,59,97]. Recently published data has shown that lung and liver are also sites of significant IL-7 production as measured by RNA message level [108]. Possibly, naïve T cells that migrate into highly vascularized organs such as liver and lung, are able to receive sufficient IL-7 survival signals that sustain them in these microenvironments. Moreover, several recent papers have shown that T cells are capable of responding to viral antigens in organs and T cells are not dependent on trafficking to draining lymph node for priming [109,110,111]. Neo-lymphoid aggregates or follicles have been described in both liver and lung, and are proposed to be sites of potent T cell immune responses [110,111].

The importance of elucidating mechanisms by which T cells, as well as other inflammatory cells, migrate into and out of the lung is underscored by the global health burden associated with lung diseases including COPD, asthma, and lung metastasis. Our data examining uninfected FtDKO mice revealed substantial increases in naïve T cells in the lung, and uncovered a selectin-ligand independent pathway of T cell migration in the absence of lung inflammation. Several years ago, another group published a study describing selectin-independent mechanisms of trafficking to the inflamed lung following *Mycobacterium tuberculosis* infection of FtDKO mice [63]. Similar to our data, they showed that activated CD8 T cell numbers were reduced in the lung early and late following infection, but these mice were able to control the infection comparable to WT mice [63]. However, the study's authors concluded that recruitment of effector T cells to lungs was solely selectin-independent, and argued that the decreased numbers they observed following infection was due to a delay in priming of T cells. Since LCMV efficiently primes T cells in the spleen, where we observed no defects in T cell migration, we conclude that our observation of decreased Ag-specific CD8 T cells in lung was likely due to the contribution of selectin-ligands in T cell homing to inflamed lung consistent with earlier work [86,88,90]. However, our study is consistent with Schreiber et. al.'s conclusion that substantial numbers of activated CD8 T cells are able to migrate into the lung and peripheral organs in a selectin ligand-independent manner [60,63].

Selectin mediated binding initiates the earliest step of lymphocyte extravasation by slowing lymphocyte rolling along HEVs. As demonstrated, the loss of selectin ligand

expression results in profound impairment in T cell extravasation to LN [62]. However, for T cell entry to the lung parenchyma, it is possible that physical slowing in the small capillaries of the lung is sufficient for T cells to efficiently enter lung routinely as part of normal migration [9,12,13]. Several studies have documented that the normal diameter of a capillary in the lung is ~7mm which requires that larger leukocytes, such as lymphocytes and granulocytes, undergo cytoskelatal rearrangment for cell deformation and elongation in order to pass through pulmonary capillary beds. Otherwise, these cells become sequestered at these sites [101,112,113]. If so, when the proportion of lymphocytes in the blood increases, such as under inflammatory conditions, or when T cell entry to the LN is blocked, T cell entry to the lung may increase as T cells become trapped in the capillary bed, and move into adjacent perivascular and intersitial compartments of the lung. Thus, selectin-mediated binding likely enhances an already effective entry process to the inflamed lung. Furthermore, additional cell adhesion molecules expressed by effector and effector memory T cells also enhance trafficking, as well as retention, in peripheral organs such as lung [114,115]. The presence of antigen and/or changes in chemokine expression in tissues following microbial infection will also influence naïve T cell migration/retention in non-lymphoid organs [116].

In summary, our data discovered that FtDKO mice had a significant increase in naïve T cells in the lungs, and demonstrated increased naïve and central memory T cell accumulation in non-lymphoid organs. Furthermore, we observe an inverse relationship between naïve T cell location in LN and non-lymphoid organs. By inhibiting T cell entry to or exit from the LN, we show that the ratio of naïve T cells in the blood is altered, and

this influences the number of naïve T cells that re-distribute to non-lymphoid organs. In patients with leukocyte adhesion deficiency diseases, where patients suffer from recurrent infections, including a significant proportion of respiratory infections, altered leukocyte trafficking may influence disease development in certain organs [117,118,119]. Proposed immunosuppressive therapies that target or alter trafficking of T cells into or out of the LN should consider that such treatments will likely influence the distribution of T cells in blood, and consequently, increase T cell accumulation in non-lymphoid organs, including the lung [120,121].

Materials and Methods

Mice and Immunizations

Fucosyltransferase -IV and -VII double knockout (FtDKO) mice, backcrossed at least 9 generations to C57BL/6 were originally generated by Dr. John Lowe [62]. Mice were obtained from the Consortium for Functional Glycomics (Scripps Research Institute, La Jolla, CA) and bred in-house as homozygous double knockouts, but breed poorly. Transgenic P14xThy1.1 mice were provided by Dr. Rafi Ahmed (Emory Vaccine Center, Atlanta, GA) and bred in-house. C57BL/6 (WT) mice were purchased from The Jackson Laboratory. Memory P14xThy1.1 chimeric mice were generated by adoptive transfer of $5x10^5$ total splenocytes from transgenic P14xThy1.1 mice into C57BL/6 recipients and infected with $2x10^5$ pfu LCMV-Armstrong. FtDKO and C57BL/6 wildtype

control mice were immunized intraperitoneally (i.p) with $2x10^5$ pfu LCMV-Armstrong. All mice were maintained under specific pathogen-free conditions at the University of Tennessee in accordance with IACUC guidelines and used at age 2 to 6 months. All animals were handled in strict accordance with good animal practice as defined by University of Tennessee IACUC committee and all animal work was approved by the committee.

Naïve and Memory Cell Trafficking

Where indicated, MACS (Miltenyi Biotech, Auburn, CA) purified naïve and memory P14xThy1.1 CD8 T cells ($2x10^6$) from the spleen were adoptively transferred into WT or FtDKO recipient mice. Naïve cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) in order to differentiate transferred naïve and memory P14xThy1.1 CD8 T cell populations. Equal numbers of Ag-specific CD8 T cells were injected intravenously (i.v.) via tail vein. In pertussis toxin (Ptx) inhibition experiments, P14xThy1.1 splenocytes were pretreated with Ptx (Calbiochem, Gibbstown NJ) or PBS for 90 minutes at 37°C [59] and $1x10^6$ Ptx or PBS treated Ag-specific CD8 T cells were adoptively transferred into WT recipient mice. For anti-CD62L experiments, 100μg of MEL-14 (α-CD62L) blocking antibody (BioXcell, W. Lebanon, NH) was injected i.v. into WT recipient mice. Four (4) hours post treatment, $1x10^6$ P14xThy1.1 CD8 T cells were injected i.v. into mice. In FTY720 experiments, $1x10^6$ P14xThy1.1 CD8 T cells were adoptively transferred into recipient WT mice, followed by treatment with 1mg/kg

FTY720 (Calbiochem) or water i.p. with treatment every 24hrs for 3 days total.

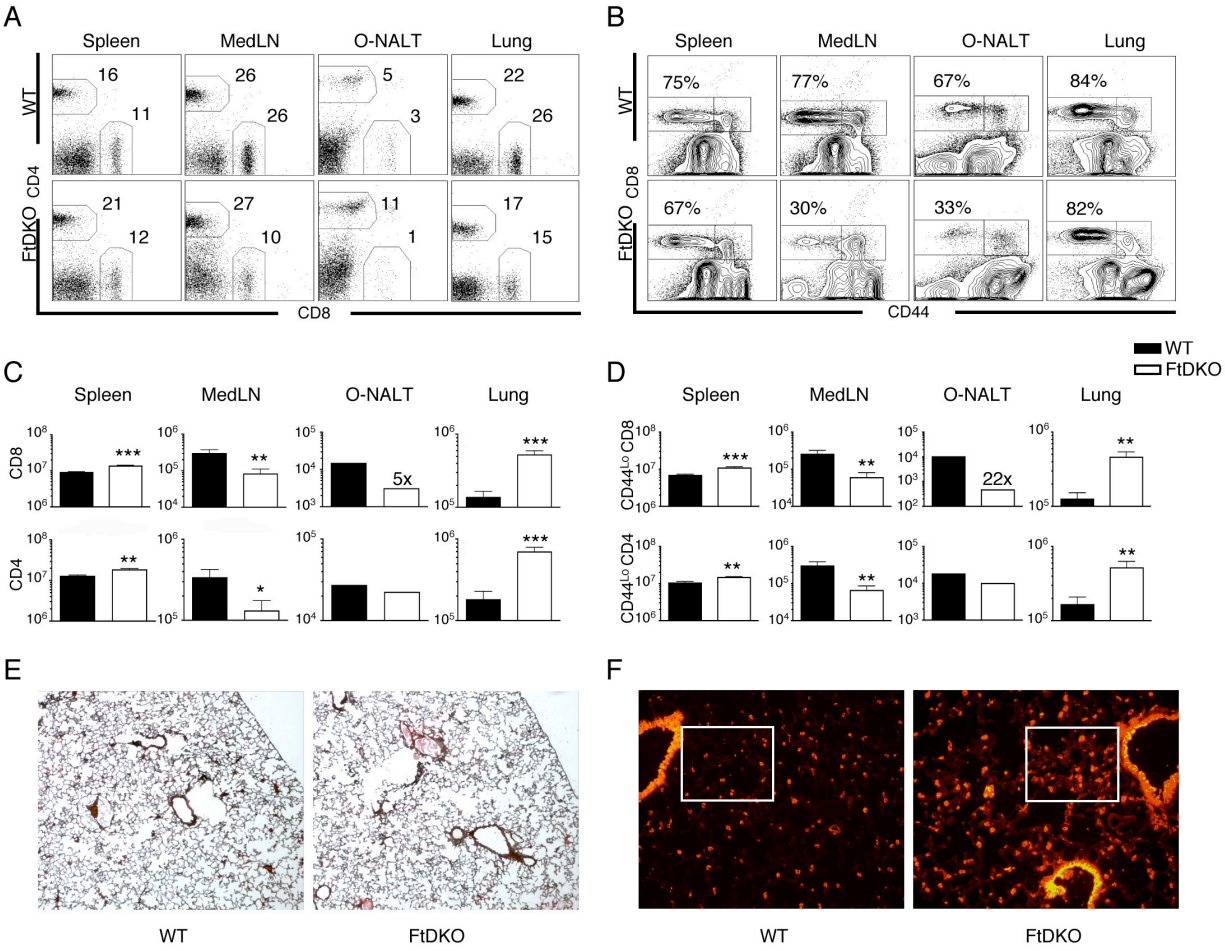
Organ Harvest, Flow Cytometry, and Histology

At indicated time points mice were sacrificed, organs were perfused with 5ml cold PBS, and tissues harvested for processing as previously described [85,122]. Briefly, perfused organs were minced, incubated with HBSS+ 1.3mM EDTA solution for 30 minutes at 37^{o}C, re-suspended in 225U/ml type I collagenase for 60 minutes at 37^{o}C, and cells were centrifuged using a Percoll density gradient to isolate lymphocytes from the parenchyma. Single cell suspensions were stained with D^{b}NP396-404 or D^{b}GP33-41 tetramers, provided by the NIH Tetramer Facility (Atlanta, GA), and mAbs (CD8, CD4, CD44, CD62L, Thy1.1, Thy1.2) [54]. Total number of cells in bone marrow was calculated by multiplying the number of cells from two femurs x 7.9 [123,124]. Numbers of cells in PBMC are shown as per 10^{6} cells. Monoclonal antibodies were purchased from BD (La Jolla, CA). All samples were run on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Treestar). For histology, lungs were infused with 1mL of 50% OCT (Tissue Tek, Torrance, CA) in PBS, embedded in OCT, and frozen on dry ice, and stored at –80^{o}C. Serial sections were cut at 7 μm and stained with hematoxylin and eosin using routine staining procedures. Lung sections were also stained with biotinylated primary Ab, Thy1.2 (1:1500; BD Biosciences), followed by Alexa Fluor 568 conjugated streptavidin (1:400; Invitrogen, Eugene, OR), and analyzed using an epifluorescent microscope (Nikon Eclipse E600). For transfer studies, to calculate the

proportions of transferred cells in different organs, total Thy1.1$^+$ CD8$^+$ T cells in indicated organs were enumerated. The number of transferred cells in each tissue was then divided by the total Thy1.1$^+$ CD8$^+$ cells in examined organs. Statistical significance was determined using unpaired student *t* tests where *indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Appendix

Figure 2.1. Increased numbers of naïve T cells in lungs of FtDKO mice. Lymphocytes were isolated from WT and FtDKO mice. *A* and *B*, Representative FACS analysis of organs from WT or FtDKO mice. Cells were stained with CD4, CD8 and CD44 mAbs. Numbers indicate percent of CD4, CD8, or $CD44^{Lo}$ CD8 T cells. *C*, Enumeration of CD8 and CD4 T cells in indicated organs. *D*, Enumeration of $CD44^{Lo}$ CD8 or $CD44^{Lo}$ CD4 T cells in indicated organs. *E*, Photomicrographs of representative H & E staining of WT and FtDKO lungs at x40 magnification. F. Photomicrographs of representative immuno histology staining of T cells using Thy1 of WT and FtDKO lungs at x200 magnification. Data was collected from n=6 WT and n=7 FtDKO in two independent experiments. For O-NALT, one experiment is shown where cells were pooled from n=4 mice/group. The unpaired Student *t* test was used to compare groups. (*$p<0.05$, ** $p<0.01$, and *** $p<0.001$)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Spleen
MedLN
O-NALT
Lung
WT
FtDKO
CD4
CD8
16
11
26
26
5
3
22
26
21
12
27
10
11
1
17
15
B
Spleen
MedLN
O-NALT
Lung
WT
FtDKO
CD8
CD44
75%
77%
67%
84%
67%
30%
33%
82%
C
Spleen
MedLN
O-NALT
Lung
CD8
CD4
***
**
*
5x
D
WT
FtDKO
Spleen
MedLN
O-NALT
Lung
CD44Lo CD8
CD44Lo CD4
22x
E
WT
FtDKO
F
WT
FtDKO

Figure 2.2. Ag-specific CD8 T cells are reduced in inflamed lung of FtDKO mice. WT or FtDKO mice were infected with LCMV-Armstrong i.p and lymphocytes were isolated on day 8 or day 60 p.i. *A*, Representative FACS analysis of single cell suspensions from indicated organs on day 8 p.i. Cells were stained with CD8, CD44 and D_bNP396-404 tetramer. Gating on CD8 T cells, numbers in FACS panels indicate percent of Ag-specific CD8 T cells in organs. *B*, Representative FACS analysis of single cell suspensions from indicated organs on day 60 p.i. Cells were stained with CD8, CD44 and D_bGP33-41 tetramer. *C*, Enumeration of Ag-specific, $CD44^{high}$, and total CD8 T cells in WT or FtDKO mice at day 8. *D*, Enumeration of Ag-specific, $CD44^{high}$, and total CD8 T cells in WT or FtDKO mice at day 60. For day 8, n=2 WT and n=3 FtDKO. For day 60, n=4 WT and n=4 FtDKO. For O-NALT, organs were pooled from each group. (*$p<0.05$, ** $p<0.01$, and *** $p<0.001$)

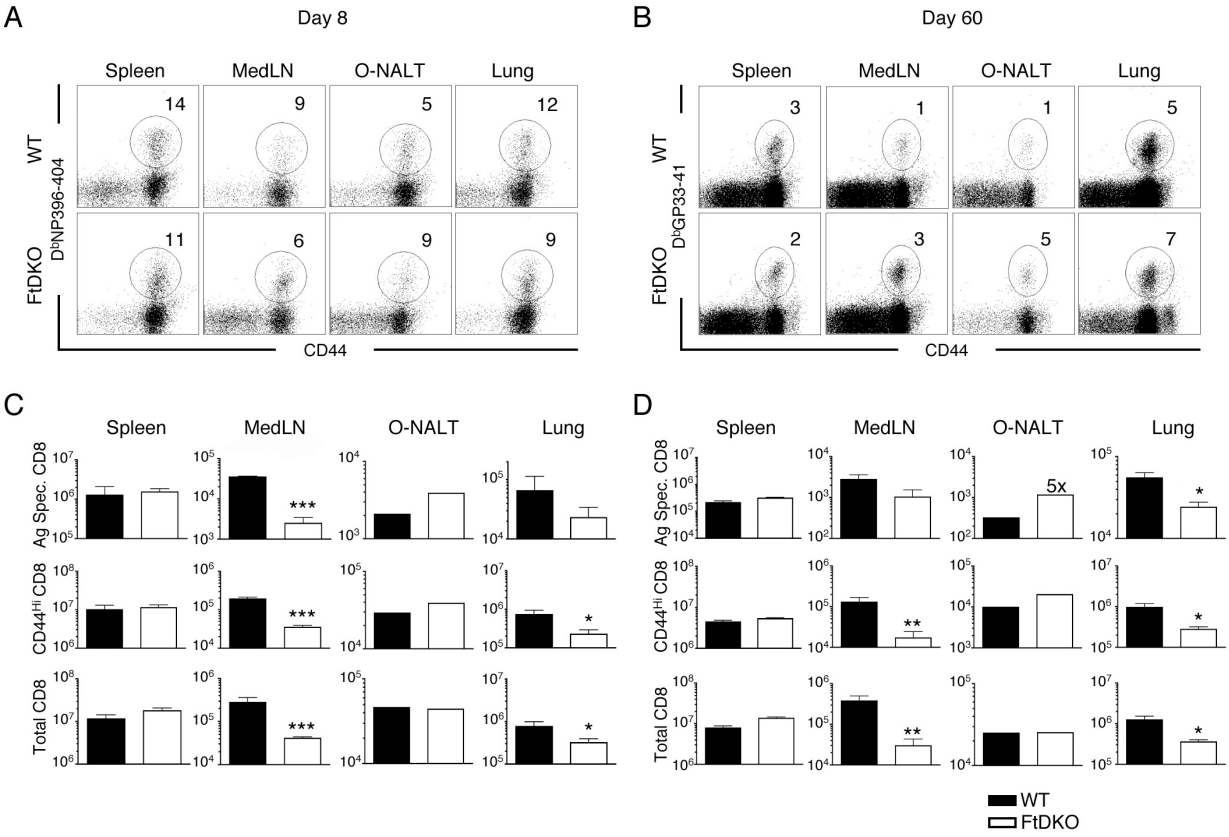
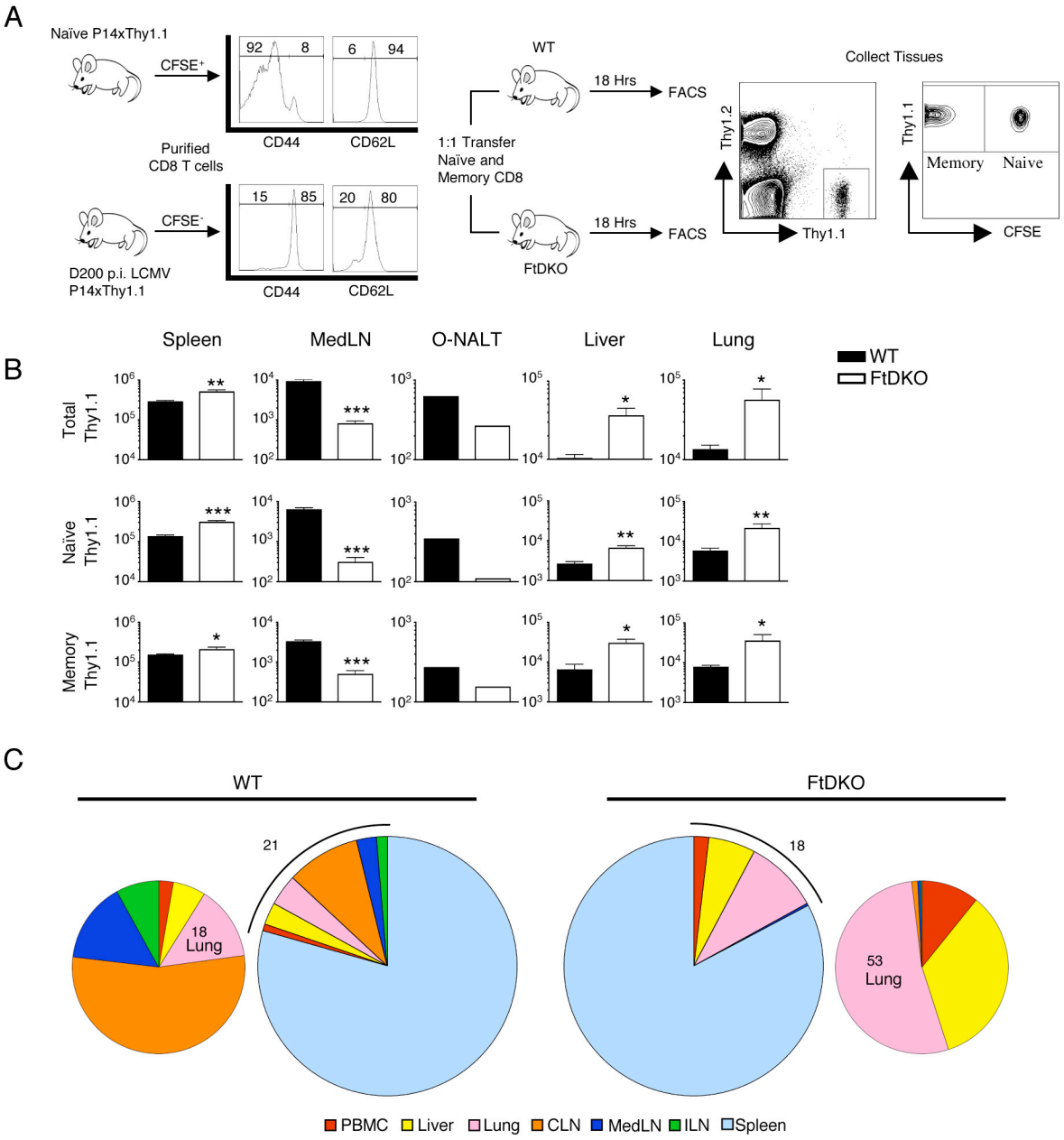

A
Day 8
Spleen
MedLN
O-NALT
Lung
WT
FtDKO
DbNP396-404
14
9
5
12
11
6
9
9
CD44
B
Day 60
Spleen
MedLN
O-NALT
Lung
WT
FtDKO
DbGP33-41
3
1
1
5
2
3
5
7
CD44
C
Spleen
MedLN
O-NALT
Lung
Ag Spec. CD8
CD44Hi CD8
Total CD8
***
***
***
*
*
*
D
Spleen
MedLN
O-NALT
Lung
Ag Spec. CD8
CD44Hi CD8
Total CD8
5x
**
**
*
*
*
WT
FtDKO

Figure 2.3. T cells preferentially accumulate in non-lymphoid organs of FtDKO mice. CD8 T cells were purified from naïve Thy1.1xP14 Tg mice ($CFSE^+$) or immune (day 200 p.i.) Thy1.1xP14 Tg chimeric mice ($CFSE^-$), transferred into WT or FtDKO recipient mice, and recipient mice were sacrificed 18 hours later. Thy1.1 was used to differentiate endogenous from transferred T cell populations from indicated organs. *A*, Experimental design and phenotype of transferred cell populations. *B*, Enumeration of transferred CD8 T cell populations in indicated organs. *C*, Pie charts showing the distribution of Thy1.1 cells in WT and FtDKO recipient mice in spleen, PBMC, liver, lung, CLN, ILN, and MedLN. Of the indicated organs, ~80% of transferred cells are located in spleen. The smaller pie chart shows the distribution of the remaining transferred cells. Data was collected from n=5 WT and n=6 FtDKO from two independent experiments. (*$p<0.05$, ** $p<0.01$, and *** $p<0.001$)


A
Naïve P14xThy1.1
CFSE+
92
8
6
94
CD44
CD62L
Purified
CD8 T cells
CFSE-
15
85
20
80
D200 p.i. LCMV
P14xThy1.1
WT
18 Hrs
FACS
1:1 Transfer
Naïve and
Memory CD8
18 Hrs
FACS
FtDKO
Collect Tissues
Thy1.2
Thy1.1
Thy1.1
Memory
Naive
CFSE
B
Spleen
MedLN
O-NALT
Liver
Lung
WT
FtDKO
Total
Thy1.1
Naïve
Thy1.1
Memory
Thy1.1
C
WT
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21
18
18
Lung
53
Lung
PBMC
Liver
Lung
CLN
MedLN
ILN
Spleen

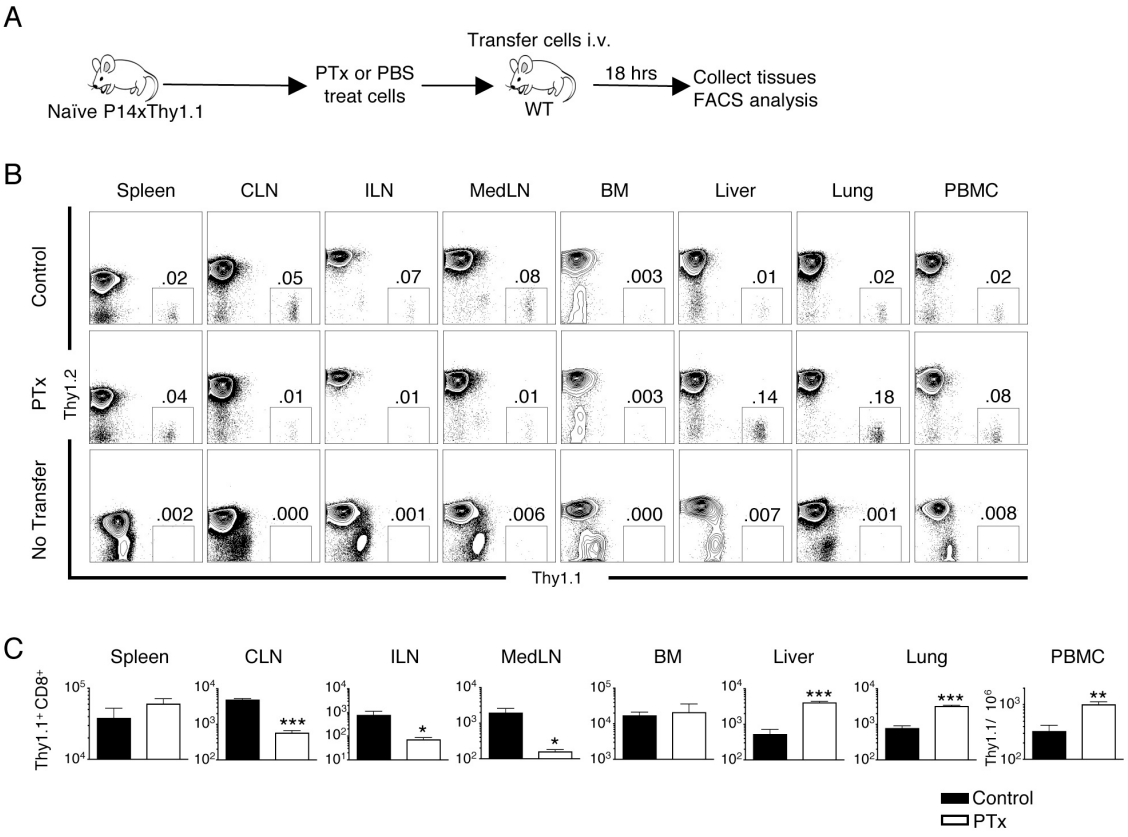


Figure 2.4. Inhibition of T cell trafficking to LN by pertussis toxin treatment results in selective re-distribution of naïve T cells to non-lymphoid organs. Splenocytes from Thy1.1xP14 Tg mice were treated with either pertussis toxin (Ptx) or PBS control and transferred into WT recipient mice. 18 hours post transfer, T cells were isolated from indicated organs and analyzed by FACS. *A*, Experimental design. *B*, Representative FACS analysis of indicated organs gating on CD8 T cells. Numbers indicate percentage of transferred cells in the indicated organ. FACS staining from animals that received untreated cells, Ptx-treated cells, or received no transferred cells are shown. *C*, Enumeration of transferred cells in organs. Data was analyzed from n=3 control untreated and n=4 Ptx treated groups. (*p<0.05, ** p<0.01, and *** p<0.001)

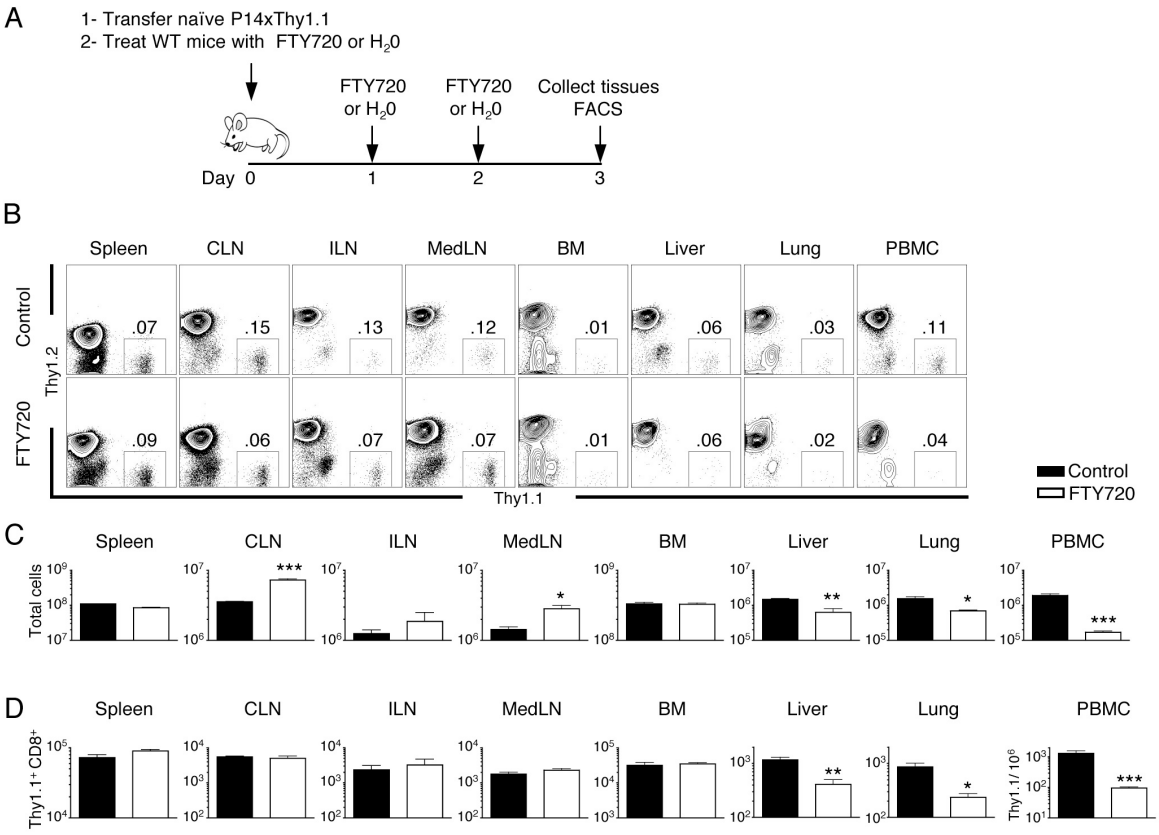
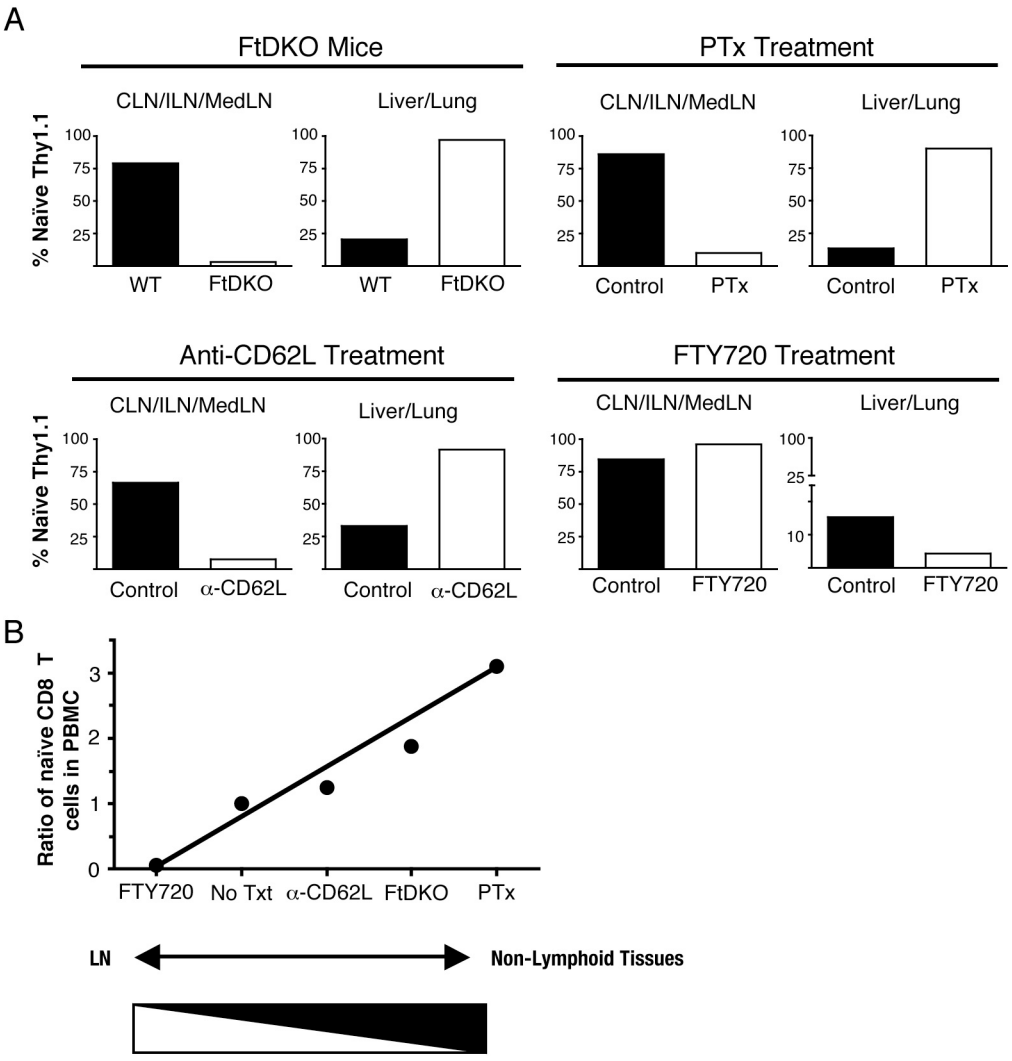


Figure 2.5. Retention of naïve T cells in LN by FTY720 treatment depletes naïve T cell populations in non-lymphoid organs. Splenocytes from naïve Thy1.1xP14 Tg mice were adoptively transferred into WT recipient. Recipient mice were treated for three days with FTY720 and lymphocytes in organs analyzed by FACS. *A*,Experimental design. *B*, Representative FACS analysis of indicated organs. Gating on CD8 T cells, numbers indicate percentage of transferred cells. *C*, Total lymphocyte numbers isolated from indicated organs. *D*, Transferred Thy1.1$^+$ CD8$^+$ T cells from indicated organs. Data from one representative experiment is shown, with n=2 control and n=3 FTY720 treated mice. (*p<0.05, ** p<0.01, and *** p<0.001)

Figure 2.6. Naïve T cell migration to non-lymphoid organs is influenced by T cell concentration in the blood. Proportions of transferred naïve T cells in selectin ligand deficient mice (FtDKO), Gαi signal disruption (Ptx), CD62L blockade, and FTY720 treated mice are shown. *A*, Total number of transferred naïve CD8 T cells in CLN, MedLN, ILN, liver, and lung was calculated, and the percent of transferred naïve CD8 T cells in LNs versus non-lymphoid organs is shown for each treatment group. *B*, The relationship of naïve CD8 T cells in the blood compared to naïve CD8 T cells in organs (LN versus non-lymphoid organs). Concentration of naïve CD8 T cells in PBMCs was determined for each animal and the ratio of treatment versus control for each treatment group is shown (R=1 for control, untreated group). Data is shown from 5 independent experiments for a total of n=28 mice.


A
FtDKO Mice
PTx Treatment
CLN/ILN/MedLN
Liver/Lung
% Naïve Thy1.1
100
75
50
25
WT
FtDKO
Control
PTx
Anti-CD62L Treatment
FTY720 Treatment
α-CD62L
FTY720
10
B
Ratio of naïve CD8 T cells in PBMC
3
2
1
0
FTY720
No Txt
α-CD62L
FtDKO
PTx
LN
Non-Lymphoid Tissues

Chapter 3

Memory T cells are enriched in lymph nodes of selectin ligand deficient mice

Research described in this chapter is a modified version of a submitted manuscript by John R. Harp, Michael A. Gilchrist, and Thandi M. Onami

Harp J. R., M. A. Gilchrist, T. M. Onami (2010) Memory CD8 T cells are enriched in lymph nodes of selectin ligand deficient mice. *J. Immunol*. Submitted

In this chapter, “our” and “we” refer to the co-authors and myself. My contribution to this paper includes: 1) planning experiments, 2) analyzing data, 3) preparing figures, and 4) editing.

Abstract

Mice deficient in expression of fucosyltransferases –IV and –VII (FtDKO) reveal profound impairment in T cell trafficking to LNs due to an inability to synthesize selectin ligands (sialyl Lewis X, sLe^x). We observed an increase in the proportion of “memory/effector” ($CD44^{high}$) T cells in LNs of FtDKO mice. We infected FtDKO mice with LCMV to generate and track Ag-specific $CD44^{high}$ CD8 T cells in secondary lymphoid organs (SLO). Although frequencies were similar, total Ag-specific effector $CD44^{high}$ CD8 T cells were significantly reduced in LNs, but not blood, of FtDKO mice at day 8. In contrast, frequencies of Ag-specific memory $CD44^{high}$ CD8 T cells were up to 8-fold higher in LNs of FtDKO mice at day 60. Since WT mice treated with anti-CD62L treatment also showed increased frequencies of $CD44^{high}$ T cells in LNs, we hypothesized that memory T cells were preferentially retained in, or preferentially migrated to, FtDKO LNs. We analyzed T cell entry and egress in LNs using adoptive

transfer of *bone fide* naïve or memory T cells. Memory T cells were not retained longer in LNs compared to naïve T cells, however, T cell exit slowed significantly as T cell numbers declined. Memory T cells were profoundly impaired in entering LNs of FtDKO mice, however, memory T cells exhibited greater homeostatic proliferation in FtDKO mice. Taken together, we conclude that FtDKO LNs are enriched in memory T cells due to longer retention because of low T cell density, and increased homeostatic expansion.

Introduction

As part of immune surveillance, T cells continuously re-circulate through lymphoid and non-lymphoid organs via the blood and lymph. The vast majority of T cells enter lymph nodes from the blood via high endothelial venules (HEVs), while a smaller fraction enters LNs through the afferent lymphatics, which drain the interstitial fluids of organs and mucosal associated lymphoid tissues (MALT). In general, it is thought that naïve T cell populations preferentially home to lymphoid organs, while memory T cells preferentially home to either bone marrow and lymphoid organs (T central memory), or non-lymphoid organs (T effector memory) [24,114,125]. However, several groups have also observed that naïve T cells regularly circulate through non-lymphoid organs such as lung, and alternative models have been proposed to account for these observations [9,33,59,105].

Selectin mediated tethering represents the earliest step in T cell entry to the LN via the HEVs. For naïve and central memory T cells this step is mediated through the

interaction of L-selectin expressed on the T cell, with selectin-ligands expressed on HEVs. L-selectin ligands are sialylated, fucosylated, and sulfated structures whose synthesis depends on the activity of several glycosyltransferases including a1,3 fucosyltransferases which are absolutely required for the synthesis of sialyl Lewis X (sLe^x) [20,73]. Mice deficient in both fucosyltransferase -IV and -VIII (FtDKO) are profoundly deficient in T cell trafficking to the LN due to loss of expression of sLe^x by HEVs [62,64]. Additionally, L-selectin ligands are further modified by the activity of two sulfotransferases, and addition of these sulfated sLe^X structures to glycoproteins such as PNAd, GlyCAM and MadCAM, allow naïve and central memory T cells to tether on HEVs, initiate chemokine dependent signaling through CCR7, and initiate firm arrest and transendothelial migration via activated integrin receptors such as LFA-1 [24,67,84].

In contrast to naïve T cells, activated T cells down-regulate L-selectin but induce expression of fucosyltransferases -IV or -VII and begin to express selectin ligands on modified T cell glycoproteins such as PSGL-1 [79,81,126]. As a consequence, activated T cells are able to interact with E- and P-selectin expressed on inflamed endothelial cells to initiate tethering and slow rolling under shear forces. Additionally, activated T cells increase expression of the adhesive proteoglycan CD44 and several integrins including VLA-4 that also contribute to slow rolling [127,128]. Moreover, studies have shown that effector/memory T cells are enriched in the afferent lymph and peripheral organs, allowing these T cells to bypass the requirement for selectin mediated trafficking by entering LNs via the afferent lymph [98,129].

Recently, we discovered that naïve T cells preferentially re-distributed and

accumulated in non-lymphoid organs such as the lung in FtDKO mice [130]. In the present study we have further examined FtDKO mice to understand how alterations in T cell trafficking to LNs affect memory T cell localization and homeostasis. Here we present our observations that FtDKO mice show a significant increase in the frequency of memory T cells in LNs, although total T cell numbers are reduced in LNs. Antibody treatment of WT mice using anti-CD62L (L-selectin) recapitulated the increase in the ratio of memory T cells in LNs. We tested whether Ag-specific $CD44^{high}$ effector or memory CD8 T cells showed alterations in trafficking to FtDKO LNs following LCMV infection. Our results demonstrated that Ag-specific memory, but not effector T cells, were significantly enriched in the LNs of FtDKO mice, even though memory T cells remain severely impaired in homing to LNs of FtDKO mice. Further, although substantial numbers of memory T cells homed to the bone marrow and spleen in both WT and FtDKO mice, T cells transferred into FtDKO mice show significantly increased homeostatic proliferation. We suggest that longer residency times of memory T cells in LNs with few lymphocytes, and increased homeostatic expansion together contribute to the preferential accumulation of Ag-specific memory T cell populations in LNs of FtDKO mice

Results

Increased frequency of CD44^{high} T cells in LNs of FtDKO mice

We isolated lymphocytes from organs of age-matched naïve WT and FtDKO mice. As has been shown previously, total cell numbers in the LNs of FtDKO mice was significantly reduced [62]. We observed a significant decrease in CD8 T cell numbers in the LN of FtDKO mice, with concomitant increases in spleen and blood (Figure 3.1B). However, analysis of the activation/memory marker CD44 on T cell populations revealed that while frequencies of CD44^{high} T cells were similar in most organs, in the LNs and mucosal associated lymphoid tissues, the frequency of CD44^{high} T cells was significantly higher in FtDKO mice (Figure 3.1A and C). Analysis of the number of CD44^{high} CD8 T cells in organs revealed that while these effector/memory CD8 T cells were generally reduced in the LN of FtDKO mice, the impairment was not as great as the reduction in total T cells. These data suggested that the impairment of trafficking of T cells to some secondary lymphoid organs (SLOs) in the absence of selectin ligands may not be as severe for effector/memory T cell populations.

Similar frequency of day 8 Ag-specific effector CD44^{high} CD8 T cells, but reduced number in LNs of FtDKO mice

We then investigated expansion and trafficking of CD44^{high} Ag-specific effector

CD8 T in lymph nodes following viral infection. WT and FtDKO mice were infected with $2x10^5$ pfu of LCMV Armstrong i.p. and Ag-specific CD8 T cells were enumerated in blood and SLOs (Figure 3.2). As expected, CD8 T cells in WT and FtDKO mice similarly down-regulate expression of CD62L by day 8 post-infection, although expression levels of CD62L were slightly elevated on naïve T cells in FtDKO mice (Figure 3.2A). Both WT and FtDKO mice show similarly high frequencies of Ag-specific $CD44^{high}$ CD8 T cells and total $CD44^{high}$ CD8 T cells in PBMCs and LNs on day 8 p.i. (Figure 3.2B, D, E). However, we observed a significant reduction in total CD8 T cell numbers in LNs of FtDKO mice compared to WT mice, and as a consequence we also observed significantly reduced Ag-specific CD8 T cells in the LNs (Figure 3.2C). Thus, while the frequencies of effector CD8 T cell populations were comparable between WT and FtDKO mice, expression of selectin-ligands remains critically important in achieving normal numbers of Ag-specific $CD44^{high}$ effector CD8 T cells in the LNs following viral infection. These data extend the findings of two previous studies using contact hypersensitivity (CHS) challenge of skin or intranasal *Mycobacterial* infection of FtDKO mice, showing loss of selectin ligands significantly impairs activated T cell migration to LNs [63,64].

Ag-specific memory $CD44^{high}$ CD8 T cells are abundant in LNs of FtDKO mice

We concluded from our analysis of effector $CD44^{high}$ CD8 T cells in LN that despite studies suggesting cooperative interaction of CD44 and VLA-4 to enable slow

rolling of activated T cell populations, T cells require expression of fucosyltransferases and synthesis of selectin ligands for activated T cell entry to the LN, as described in earlier studies [79,81,128]. In contrast to our findings with Ag-specific effector CD8 T cells at day 8, our analysis of Ag-specific memory CD8 T cell populations in LNs of immune FtDKO mice revealed significant increases in the frequencies of memory cells compared to WT mice at day 60, (Figure 3.3A, C). Similar to our observations in uninfected FtDKO mice, in day 60 immune mice, the frequency of $CD44^{high}$ CD8 T cell populations was increased in LNs of FtDKO mice (Figure 3.3E). Importantly, despite our continued observation that total CD8 T cells remained substantially reduced in LNs of FtDKO mice, the significantly higher frequency of Ag-specific memory CD8 T cells in the LNs resulted in comparable numbers of Ag-specific memory CD8 T cells in LNs of WT and FtDKO mice (Figure 3.3B and D). Thus, despite the loss of selectin ligands, Ag-specific memory T cells were similarly abundant in the LNs of FtDKO mice.

There were several possible explanations for our results demonstrating increased frequencies of memory T cells in the LNs of FtDKO mice. T cell density has been associated with T cell survival, and it was possible that there was increased survival of memory T cells in LNs of FtDKO mice. However, studies that have examined T cell survival and density have found that high T cell density favors survival, while low T cell density results in increased apoptosis [131]. Therefore, we initially considered two explanations to test experimentally. One possible explanation was that in contrast to naïve or effector CD8 T cell populations, Ag-specific memory CD8 T cells were being preferentially retained in LNs of FtDKO mice. A second hypothesis was that memory T

cells were preferentially homing to the LNs of FtDKO mice via selectin ligand independent mechanisms.

Ratio of Memory T cells increases in WT mice following inhibition of L-selectin mediated entry

To test whether inhibition of T cell entry changed the ratio of naïve versus memory T cell populations, we treated normal mice with anti-CD62L. As expected, treatment of WT mice with anti-CD62L resulted in a significant reduction in total CD8 T cell numbers in the LNs, but not spleen (Figure 3.4A). However, this reduction in total CD8 T cell numbers was almost entirely due to a decrease in naïve $CD44^{low}$ CD8 T cells (Figure 3.4A). In contrast, the memory/effector $CD44^{high}$ population was relatively stable, and we observed a significant increase in the percentage of $CD44^{high}$ CD8 T cells in LNs, similar to our observations in FtDKO mice (Figure 3.4A, B). Similar results were observed with the CD4 T cell population (data not shown). Thus blockade of T cell entry via the L-selectin mediated HEV-dependent pathway results in the increased frequency of endogenous effector/memory $CD44^{high}$ T cell populations in LNs similar to the observed increases in FtDKO mice (Figure 3.1). This observation was consistent with data presented in an elegant recent study which suggested that memory T cells were retained for longer periods in LNs [132]. However, in both studies, T cells were activated by an unknown, endogenous antigen, and we cannot determine what proportion of the $CD44^{high}$ memory/effector population was proliferating in response to

endogenous antigen present in the LN. Thus, whether memory T cells in the absence of specific antigen show differences in retention in LN was unclear.

Exit rates of naïve and memory CD8 T cells from LN are similar, but residency time in the LN is density-dependent

To directly test the hypothesis that memory T cells were retained longer in the LNs compared to naïve T cells, we transferred *bone fide* Thy1.1^{+} naïve or memory CD8 T cells with the same TCR specificity into WT Thy1.2^{+} recipient mice. We used the P14 transgenic TCR specific for the GP33-41 peptide of LCMV. Both populations of Thy1.1^{+} CD8 T cells were CD62L^{high} and either CD44^{low} (naïve, antigen-inexperienced), or CD44^{high} (memory, antigen-experienced). Following transfer into recipient mice, further T cell entry was blocked with anti-CD62L treatment of mice to measure the decay in transferred T cell populations in different organs over time. We then calculated the exit rate of these T cell populations. This evaluation allowed us to determine whether memory T cells show differences in residency time in LNs compared to naïve T cells.

There was one zero cell count for both the memory and naive cell populations. Both zeros occurred in the MedLN at 48 hours but in separate mice. Because we were working with log-transformed data, these zero counts were replaced with the lowest non-zero count for the entire dataset (13.2 cells). The best fit model was the one which assumed that egress rates differed between the early, 0 to 8 hours, and the late, 24 to 48 hours, time periods ($F = 21.035$, $df = 1$, p-value $< 10^{-4}$), but where the exit rate was

the same for both memory and naive cell types within each time frame ($F = 0.0682$, df = 2, p-value >0.9) for all LNs except for the late ILN treatment ($F = 9.1651$, df = 1, p-value <0.005). The egress parameter estimates are summarized in Table 3.I.

Our analysis revealed that naïve and memory CD8 T cells showed no statistical differences in exit rates from the LNs (Figure 3.5). Initially, both naïve and memory CD8 T cell populations show residency times of about 5-6 hrs in LNs, with memory T cells showing a slight trend towards faster exit. However, our data also revealed that as the density of lymphocyte populations decreased in the LN over the treatment time (due to anti-CD62L blockade of new lymphocyte entry to LNs with continued cell exit), the rate of T cell egress significantly slowed for both naïve and memory T cell populations to about 15-16 hrs in some LNs, to as much as 84 hrs (3.5 days) in others (Table 3.I). Several studies examining T cell recirculation have suggested that T cell migration through the T cell area of the LN is slowed when T cell density is reduced, resulting in reduced exit and increased T cell retention [133,134,135]. While undoubtedly in our data set the relative contribution from non-HEV dependent T cell entry increased as the overall HEV-dependent contribution decreased, our data is consistent with this earlier work measuring thoracic duct T cells in irradiated and nude animals which showed that density of T cells in LNs influences T cell retention [133,134,135]. Further, this data suggested that since endogenous T cell populations are quite low in LNs of FtDKO mice, T cells that enter LNs of FtDKO mice would be retained longer relative to T cells that enter LNs of WT mice since T cell density is up to 95% higher in LNs of WT mice.

Memory T cells are significantly impaired in trafficking to LNs of FtDKO mice

Since exit rates of naïve and memory T cells were similar in our study, we tested our second hypothesis that memory T cells were selectively homing to the LNs of FtDKO mice. We transferred *bone fide* Thy1.1^{+} naïve or memory CD8 T cells that were specific for the GP33-41 peptide of LCMV into WT or FtDKO Thy1.2^{+} recipient mice. As before, both populations of Thy1.1^{+} CD8 T cells were CD62L^{high} and either CD44^{low} (naïve, antigen-inexperienced), or CD44^{high} (memory, antigen-experienced). Additionally, since these T cells were derived from WT mice, they retained the ability to express fucosyltransferases following activation and make sLe^{x} bearing selectin ligands, and could therefore potentially interact with E- or P-selectin expressed on HEVs of both WT and FtDKO mice. Contrary to our hypothesis, transfer of memory CD8 T cells into FtDKO mice also revealed a profound impairment in homing to LNs (Figure 3.6). While in both WT and FtDKO mice, the majority of transferred memory T cells migrated to the bone marrow and spleen, the remaining memory CD8 T cells preferentially homed to the LNs in WT mice with very few cells homing to the LNs in FtDKO mice (51% WT vs. 2% FtDKO). In contrast, in the FtDKO mice, the memory T cells that did not home to bone marrow or spleen preferentially homed to the lung and liver (89% FtDKO).

These data strongly argue that overall, memory T cells remain significantly impaired in homing to the LNs of FtDKO mice compared to WT mice (Figure 3.6B, C). Further, they suggest memory T cells also require selectin ligand expression by HEVs for efficient homing to LNs. However, despite significant reductions in the total number

of T cells that homed to the LNs of FtDKO mice, our analysis also revealed that comparing memory versus naïve T cell populations, the small population of transferred T cells that did migrate to LNs in FtDKO mice were more likely to be memory T cells compared to WT mice (Figure 3.6E). Specifically, in WT mice we observed about a 3:1 preferential homing of naïve to memory T cells in LN, while in FtDKO mice we observed no preferential homing of naïve T cells (Figure 3.6D, E). In fact, the frequency of memory T cells was slightly increased in the FtDKO mice (Figure 3.6E). Stated another way, these data suggest that entry of T cells into the LN via the non-HEV dependent pathway (i.e. the afferent lymph) may be similar for naïve and memory T cells, while HEV dependent entry strongly favors naïve T cells, even when memory T cells are $CD62L^{high}$.

Memory T cells show increased homeostatic proliferation and accumulation in FtDKO mice

Our data indicated that memory T cells were not preferentially retained in LNs compared to naïve T cells, and also showed memory T cells did not preferentially migrate to LNs of FtDKO mice. However, despite significant impairment in migration of both naïve and memory T cell populations to LNs, the ratio of transferred memory T cells was higher in FtDKO mice compared to WT mice. Since T cell populations are substantially reduced in FtDKO LNs, our studies examining T cell exit suggested that the few T cells that entered the low T cell density LNs of FtDKO mice would be retained

longer at these sites. We therefore proposed an alternate hypothesis that memory T cell populations that entered and exited LNs of FtDKO mice would show increased homeostatic proliferation, and memory T cells would preferentially accumulate in LNs over time.

To test this, we labeled P14xThy1.1^{+} memory CD8 T cells with CFSE and transferred them into WT and FtDKO mice. Three weeks later we isolated organs from recipient mice and assessed proliferation by CFSE dilution. As shown in Figure 3.7, memory T cells transferred into FtDKO mice show greater homeostatic proliferation compared to memory T cells transferred into WT mice (Figure 3.7A). Moreover, we also observed that significantly more memory T cells were found in LNs of FtDKO mice at day 21 compared to day 1 (Figure 3.7B). As before, the majority of transferred memory T cells were located in bone marrow and spleen of WT and FtDKO recipient mice (~88%). Of the remaining transferred cells, a greater fraction of memory T cells were found in the LNs of FtDKO mice at day 21 compared to day 1 after transfer (Figure 3.7B). Whereas, day 1 after transfer into recipient mice, only 2% of transferred memory T cells were found in LNs of FtDKO mice, by day 21 the percentage of memory T cells observed in LNs increased more than 15-fold, to 35% (Figures 3.7B and 3.6B). Taken together, these data suggest that memory T cells are enriched and remain abundant in the LNs of FtDKO mice, despite severely impaired trafficking to LNs, as a consequence of several mechanisms of T cell homeostatic maintenance, including longer retention in low cell density LNs and increased homeostatic proliferation.

Discussion

The generation of effective immune responses is critically dependent on encounters between re-circulating T cells and their specific antigens, which primarily occur in secondary lymphoid organs. One of the defining characteristics of the immune system is the constant movement of constituent cell populations, including T cells and dendritic cells (DCs) through SLOs, where a highly efficient filtration and surveillance system are optimized for the capture of antigens and appropriate antigen presentation [136,137,138]. Further, in contrast to naïve T cells, memory T cells respond more rapidly to specific antigen, based on both quantitative increases in their starting population, and qualitative differences in effector responses including rapid cytokine secretion [139].

In this study, we present evidence that FtDKO mice, that are incapable of synthesizing sLe^x bearing-selectin ligands, show significant enrichment of endogenous $CD44^{high}$ effector/memory T cell populations in LNs and MALT such as O-NALT (Figure 3.1). When we infected FtDKO mice with LCMV to track Ag-specific $CD44^{high}$ CD8 T cell responses in LNs, we show similar responses in blood and spleen of mice at day 8, but Ag-specific $CD44^{high}$ CD8 T cells were reduced in LNs (Figure 3.2). Thus, although Ag-specific CD8 T cells increased significantly in LNs of FtDKO mice at day 8 compared to naïve FtDKO mice, selectin ligands were required for efficient Ag-specific CD8 T cell trafficking and expansion in LNs. These data show for the first time that Ag-specific T cell responses remain compromised in LNs following viral infection of these mice, even

though these mice show similar kinetics of viral clearance [60].

In contrast, to our observations at day 8 following viral infection, we observed that at day 60 p.i. FtDKO mice demonstrated abundant Ag-specific CD8 T cell responses both in spleen and LNs (Figure 3.3). While total CD8 T cells remained substantially reduced in LNs of FtDKO mice, the higher frequencies of Ag-specific memory CD8 T cells in the LN compensated for the overall low T cell numbers and particularly low $CD44^{low}$ naïve T cell population (Figure 3.3). These data suggest that selectin ligands are not required for efficient localization of Ag-specific memory CD8 T cells in LNs of immune mice.

We can recapitulate a similar increase in the percentage of $CD44^{high}$ T cells in LN by treating WT mice with anti-CD62L, which substantially reduced LN T cell numbers by blocking entry of new lymphocytes (Figure 3.4). Our analysis of T cell exit rates from LN revealed no significant differences comparing naïve and memory T cells (Figure 3.5). While these data contrast with a recent study examining *in vivo* $CD44^{high}$ endogenous CD4 T cell populations using Kaede-Tg mice, several differences in the two studies must be considered [132]. Firstly, we examined memory T cells of known antigen specificity, while the Tomura et. al study examined endogenous $CD44^{high}$ T cells. When we examined endogenous $CD44^{high}$ T cell populations, we also observed stable numbers of this population, while naïve $CD44^{low}$ T cell numbers plummeted (Figure 3.4). In our experiment, this could be due to continued entry of $CD62L^{low}$ $CD44^{high}$ T cells, slower exit of the $CD44^{high}$ population, or conversion of $CD44^{low}$ to $CD44^{high}$ T cells. However, since we cannot determine whether these $CD44^{high}$ cells have recently

encountered antigen, we cannot conclude that activated/memory T cells not exposed to specific antigen were retained for longer periods based on these data. We therefore examined *bona fide* Ag-specific memory T cells, where analysis of egress experiments suggested that naïve T cells do not exit faster than memory T cells (Figure 3.5). Also, while our analysis focused on CD8 T cells, the Tomura et al study focused on CD4 T cells, which interact with a limited population of Ag-presenting cells expressing MHC class II. Since memory CD4 T cells also show differences in survival and maintenance compared to memory CD8 T cells, it is possible that memory CD4 T cells are indeed retained longer in LN, as this study concluded, whereas memory CD8 T cell retention is regulated differently [132,140].

Importantly, T cell density influences T cell exit rates from the LN. Our data suggested that exit rates slowed as T cell density decreased (Figure 3.5). A previous study suggested that T cells that enter LNs with many cells exit faster (ie shorter residence time), while T cells that enter LNs with fewer cells, exit slower (ie longer residency times) and our study examining Ag-specific naïve and memory T cells is consistent with this study [135]. Further, when we examined memory T cell trafficking into the LNs of FtDKO mice, we discovered that both naïve and memory T cells were significantly impaired in homing to the LNs of FtDKO mice following adoptive transfer (Figure 3.6). Thus the enrichment of memory T cells in the LNs of immune FtDKO mice is unlikely to be due to significantly increased selectin-independent entry of memory T cell populations into LNs.

The distribution of memory T cells in different organs will be different at different

time-points since T cell migration is a very dynamic process dependent on the expression of several adhesion molecules and chemotactic responses [141,142]. Our experiments examining day 1 post-transfer showed that for both WT and FtDKO mice, a significant fraction of memory T cells homed to the bone marrow and spleen (Figure 3.6). These results are consistent with several studies that have demonstrated that memory T cells preferentially reside in the bone marrow where they undergo homeostatic proliferation or proliferative renewal due to IL-15 maintenance signals [105,123,143,144]. In contrast to WT mice, where the majority of the remaining CD62L^{high} memory T cells homed to the LNs, most of the transferred memory T cells were located in non-lymphoid organs in FtDKO mice (Figure 3.6B). We have recently demonstrated that naïve T cells also preferentially re-distribute to non-lymphoid organs such as lung in FtDKO mice [130]. We conclude from these studies that naïve, effector, and memory CD8 T cell populations all show dependence on the expression of selectin ligands for homing to LNs. However, we also observed that frequencies of effector/memory (CD44^{high}) T cells were consistently enriched in FtDKO mice, except at day 8 when both WT and FtDKO showed similarly high proportions of CD44^{high} CD8 T cells of 60-90% (Figure 3.2).

Even though entry of memory T cells into LN was significantly impaired, the few T cells that entered LNs of FtDKO mice, were more likely to be memory T cells compared to LNs of WT mice, where naïve T cells preferentially homed (Figure 3.6). Since these memory T cells entered LNs with very low T cell density in FtDKO mice, we considered that longer retention in these organs could trigger greater homeostatic

proliferation, and our observations confirm this hypothesis (Figure 3.7). In lymphoid organs, DCs and stromal cells such as fibroblastic reticular cells (FRCs) form a complex three-dimensional meshwork through which T cells and other lymphocytes must travel [137]. T cells that enter the depleted LNs of FtDKO mice could undergo lymphopenia-induced proliferation in the T cell zone if retained for a sufficient length of time at these locations. However, antigen-independent T cell proliferation occurs more slowly than antigen-driven proliferation and we were uncertain if the retention times in LN would be sufficient for lymphopenia induced proliferation to occur. Additionally, most memory T cells were located in bone marrow and spleen, major sites of memory T cell proliferation, and we observed no defects in migration to these locations following transfer into FtDKO mice [123]. Nonetheless, our data clearly demonstrated that memory T cells divided more when transferred into fucosyltransferase deficient mice (Figure 3.7). Thus, we suggest that the enrichment of memory CD8 T cells in LNs of FtDKO mice compared to WT mice is due to several homeostatic maintenance mechanisms. These include increased entry of memory T cells via non-HEV dependent pathways (afferent lymphatics) compared to HEV dependent entry which favors naïve T cells, slower exit of T cells from LNs with low T cell density, and increased homeostatic proliferation of T cells. Together, these mechanisms enable abundant populations of memory T cells to reside in lymph nodes and mucosal associated tissues of mice lacking a critical glycosylation-dependent determinant of cell adhesion molecules, even though migration of naïve, effector, and memory T cells to these critical secondary lymphoid organs, is severely compromised.

Materials and Methods

Mice and Immunizations

Fucosyltransferase -IV and -VII double knockout (FtDKO) mice backcrossed at least 9 generations to C57BL/6 were originally generated by Dr. John Lowe [62]. Mice were obtained from the Consortium for Functional Glycomics (Scripps Research Institute, La Jolla, CA) and originally bred in-house as homozygous double knockouts, but breed poorly, and are currently maintained as heterozygous breeding pairs and genotyped by PCR. Transgenic P14xThy1.1 mice were provided by Dr. Rafi Ahmed (Emory Vaccine Center, Atlanta, GA) and bred in-house. C57BL/6 (WT) mice were purchased from The Jackson Laboratory. Memory P14xThy1.1 chimeric mice were generated by adoptive transfer of $5x10^5$ total splenocytes from transgenic P14xThy1.1 mice into C57BL/6 recipients and infected with $2x10^5$ pfu LCMV-Armstrong. FtDKO and C57BL/6 wildtype control mice were immunized intraperitoneally (i.p) with $2x10^5$ pfu LCMV-Armstrong. All mice were maintained under specific pathogen-free conditions at the University of Tennessee in accordance with IACUC guidelines and used at age 2 to 6 months.

Naïve and Memory Cell Trafficking

Where indicated, MACS (Miltenyi Biotech, Auburn, CA) purified naïve and memory P14xThy1.1 CD8 T cells ($1\text{-}2x10^6$) from the spleen were adoptively transferred into WT

or FtDKO recipient mice (Thy1.2). Naïve or memory cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) in order to differentiate transferred naïve and memory P14xThy1.1 CD8 T cell populations, or to examine homeostatic proliferation. Equal numbers of Ag-specific CD8 T cells were injected intravenously (i.v.) via tail vein into age-matched recipient mice. For anti-CD62L experiments to measure exit rates, equal numbers of naïve and memory T cells were adoptively transferred into recipient mice, and one day later 100μg of MEL-14 (α-CD62L) blocking antibody (BioXcell, W. Lebanon, NH) was injected i.v. Organs were collected before treatment and 8, 24, and 48 hours after treatment, and exit rates were calculated as indicated below.

Organ Harvest, Flow Cytometry, and Histology

At indicated time points mice were sacrificed, organs were perfused with 5ml cold PBS, and all organs were harvested for processing as previously described [85,122]. Briefly, for lung and liver, perfused organs were minced, incubated with HBSS+ 1.3mM EDTA solution for 30 minutes at 37^{o}C, re-suspended in 225U/ml type I collagenase for 60 minutes at 37^{o}C, and cells were centrifuged using a Percoll density gradient to isolate lymphocytes from the parenchyma. Isolated lymphocytes were counted from all organs and single cell suspensions were stained with D^{b}NP396-404 or D^{b}GP33-41 tetramers, provided by the NIH Tetramer Facility (Atlanta, GA), and mAbs (CD8, CD4, CD44, CD62L, Thy1.1, Thy1.2) [145]. Total number of cells in bone marrow was calculated by

multiplying the number of cells from two femurs x 7.9 [123]. Monoclonal antibodies were purchased from BD (La Jolla, CA). All samples were run on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Treestar).

For transfer studies, to calculate the proportions of transferred cells in different organs, total, naïve, or memory Thy1.1$^+$ CD8$^+$ T cells in 9 indicated organs were enumerated. The number of transferred memory cells in each organ was then divided by the memory cell populations in all examined organs as depicted by a pie graph. Statistical significance was determined using unpaired student *t* tests where *indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Modeling of naïve and memory exit rates

Our analysis is based on the following, simple model of T cell inflow into a lymph node,

$$\frac{dT}{dt} = \lambda(t) - \varepsilon\, T(t) \qquad (1)$$

where $T(t)$ is the density of a given CD8+ cell type, naive or memory, at time t, $\lambda(t)$ is the immigration rate of these cells into a given LN and ε is the egress rate. Assuming that the drug treatment results in $\lambda(t)$ being reduced to near zero values, Equation (1) implies that $T(t)$ will undergo an exponential decay process. That is, given a cell density at some time t_i, $T(t_i)$, the density of cells at time $t + \tau$ is simply,

$$T(t_i + \tau) = T(t_i)e^{-\varepsilon\tau}. \quad (2)$$

Taking the log of both sides of Equation (2) yields,

$$\ln\big(T(t_i + \tau)\big) = \ln\big(T(t_i)\big) - \varepsilon\,\tau.$$

Thus, we can estimate ε at different time points by fitting a linear regression model to the log transformed data at times t_i and $t_i + \tau$. Statistical tests for differences in ε as a function cell type, tissue, and time period using an F-test. Specifically, for the Early treatment $t_i = 0\,h$ and $\tau = 8\,h$ and for the Late treatment $t_i = 24\,h$ and $\tau = 24\,h$. Regression fits to the log transformed data and F-statistic calculations and comparisons were done in R 2.11 using the the lm and anova functions which are part of the R's standard statistical package stats.

Appendix

Figure 3.1. Increased frequency of CD44^{high} T cells in LNs of FtDKO mice. Lymphocytes were isolated from organs of WT and FtDKO mice. Cells were stained with CD4, CD8, and CD44 mAbs. Numbers indicate percentage of CD44^{high} CD8 T cells. *A*, Representative FACS analysis of indicated organs from WT or FtDKO mice. *B,* Enumeration of total CD8 T cells and CD44^{high} CD8 T cells in indicated organs. *C*, Percentage of CD44^{high} CD8 T cells in indicated organs. Data was collected from n=6 WT and n=6-7 FtDKO in two independent experiments. For O-NALT, one experiment is shown where cells were pooled from n=4 mice/group. The unpaired Student *t* test was used to compare groups. (*$p<0.05$, ** $p<0.01$, and *** $p<0.001$)

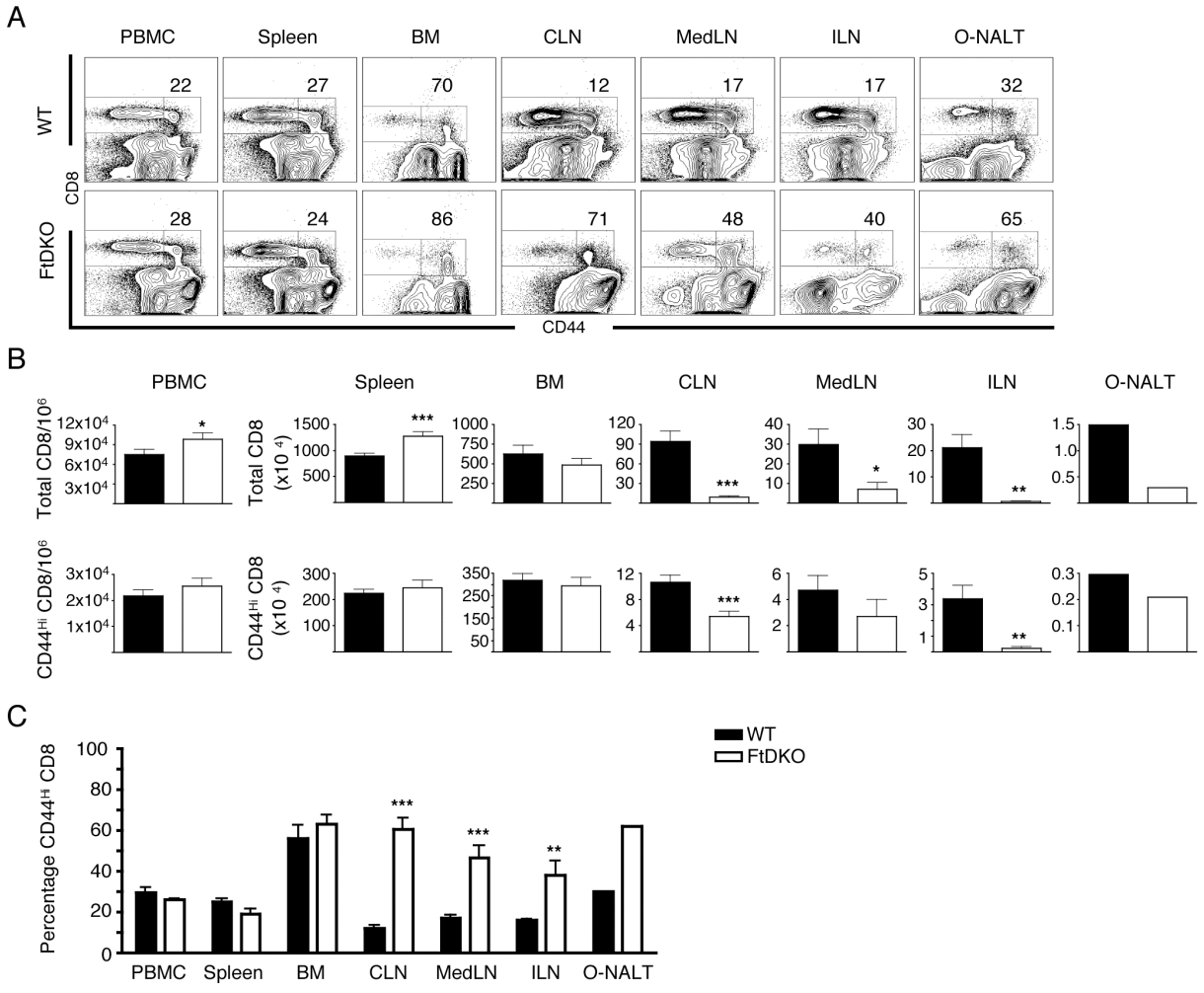

A
PBMC
Spleen
BM
CLN
MedLN
ILN
O-NALT
WT
FtDKO
CD8
CD44
22
27
70
12
17
17
32
28
24
86
71
48
40
65
B
Total CD8/10^6
Total CD8 (x10^4)
CD44^Hi CD8/10^6
CD44^Hi CD8 (x10^4)
C
Percentage CD44^H CD8
WT
FtDKO

Figure 3.2. Similar frequency of day 8 Ag-specific effector $CD44^{high}$ CD8 T cells, but reduced number in LNs of FtDKO mice. WT or FtDKO mice were infected with $2x10^5$ pfu LCMV-Armstrong i.p. and lymphocytes were isolated on day 8. Single cell suspensions from indicated organs were stained with CD8, CD44, and D_bNP396-401 tetramer. Gating on CD8 T cells, numbers in FACS panels indicate percentage of Ag-specific CD8 T cells in organs. *A*, Histogram showing expression of CD62L on CD8 T cells on day 0 and 8 in WT and FtDKO mice. *B*, Representative FACS analysis of PBMCs and LNs on day 8 p.i. *C*, Enumeration of Ag-specific and total CD8 T cells on day 8. *D*, Ratio of Ag-specific CD8 T cells, comparing FtDKO vs. WT mice in indicated organs. *E*, Percentage of $CD44^{high}$ CD8 T cells in indicated organs. Data was collected from n=2 WT and n=3 FtDKO. (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$)

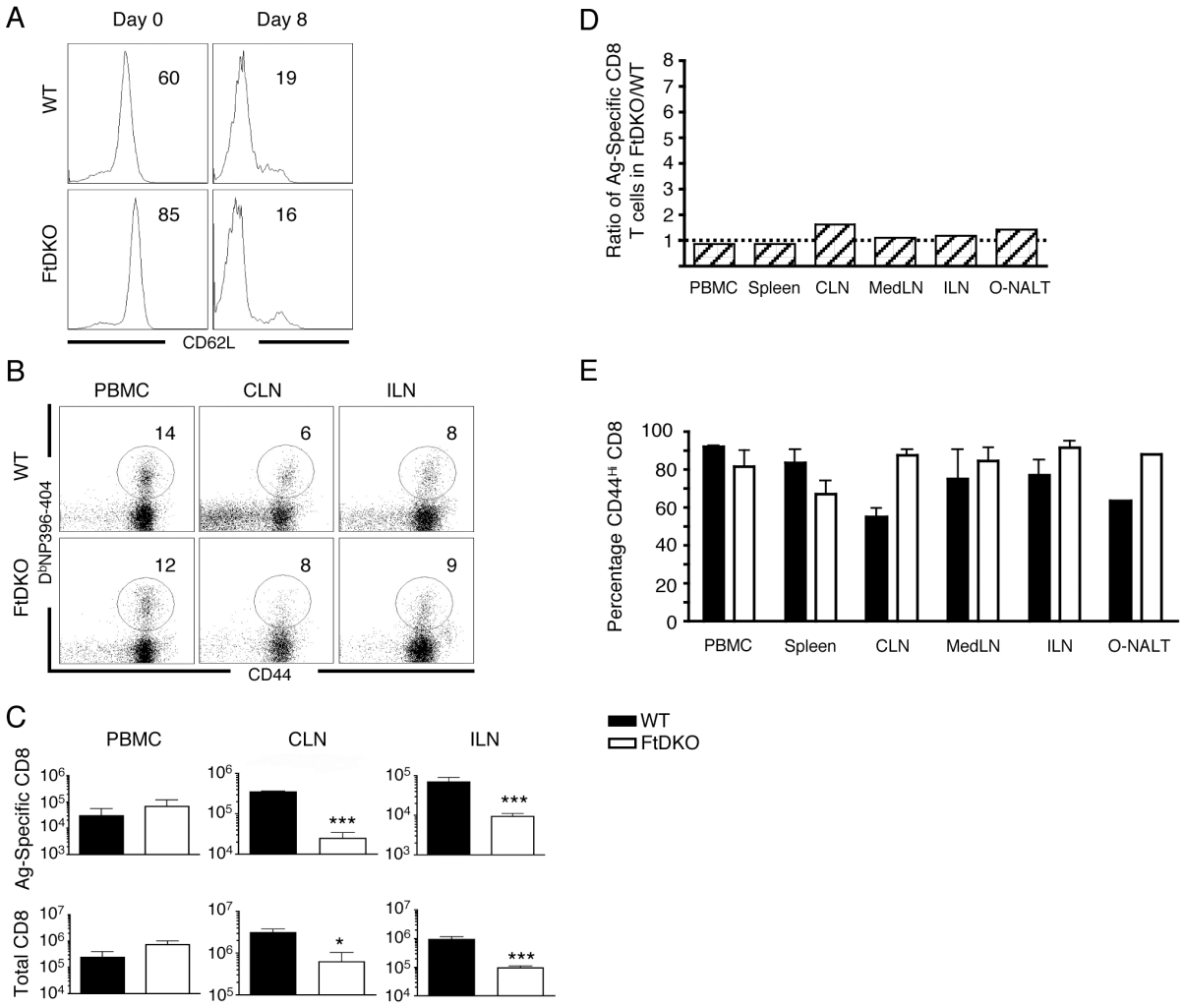
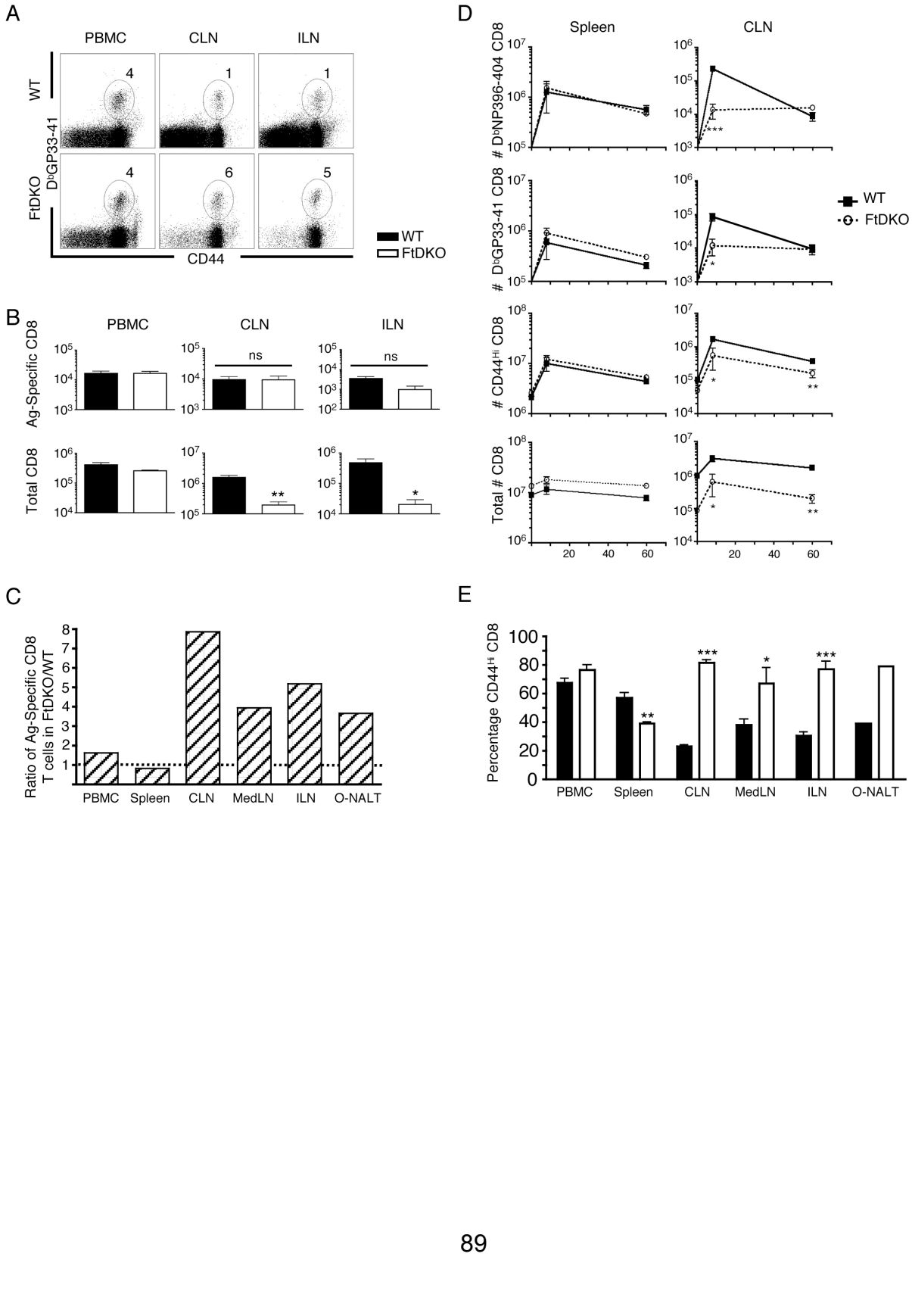

A
Day 0
Day 8
WT
FtDKO
60
19
85
16
CD62L
B
PBMC
CLN
ILN
WT
FtDKO
DbNP396-404
14
6
8
12
8
9
CD44
C
PBMC
CLN
ILN
Ag-Specific CD8
Total CD8
***
*
***
D
Ratio of Ag-Specific CD8 T cells in FtDKO/WT
PBMC
Spleen
CLN
MedLN
ILN
O-NALT
E
Percentage CD44H CD8
PBMC
Spleen
CLN
MedLN
ILN
O-NALT
WT
FtDKO

Figure 3.3. Ag-specific memory CD44^{high} CD8 T cells are abundant in LNs of FtDKO mice. WT or FtDKO were infected with LCMV-Armstrong i.p. and lymphocytes were isolated on day 60 p.i. Single cell suspensions were stained with CD8, CD44, and D_bGP33-41 tetramer. Gating on CD8 T cells, numbers in FACS panels indicate percent of Ag-specific CD8 T cells in organs. *A*, Representative FACS analysis Of PBMC and LNs on day 60. *B*, Enumeration of Ag-specific and total CD8 T cells on day 60. *C*, Ratio of Ag-specific CD8 T cells, comparing FtDKO vs. WT mice in indicated organs. D, Time-course following LCMV-Armstrong infection of D_bNP396-401-specific CD8 T cells, D_bGP33-41-specific CD8 T cells, CD44^{high} CD8 T cells, and total CD8 T cells comparing spleen and CLN. *E*, Percentage of CD44^{high} CD8 T cells in organs. Data was collected from n=4 WT and n=4 FtDKO. (*$p<0.05$, ** $p<0.01$, *** $p<0.001$, and ns, not significant)


A
PBMC
CLN
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WT
FtDKO
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B
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Ag-Specific CD8
Total CD8
ns
ns
**
*
C
Ratio of Ag-Specific CD8 T cells in FtDKO/WT
PBMC
Spleen
CLN
MedLN
ILN
O-NALT
D
Spleen
CLN
DbNP396-404 CD8
DbGP33-41 CD8
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E
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CLN
MedLN
ILN
O-NALT

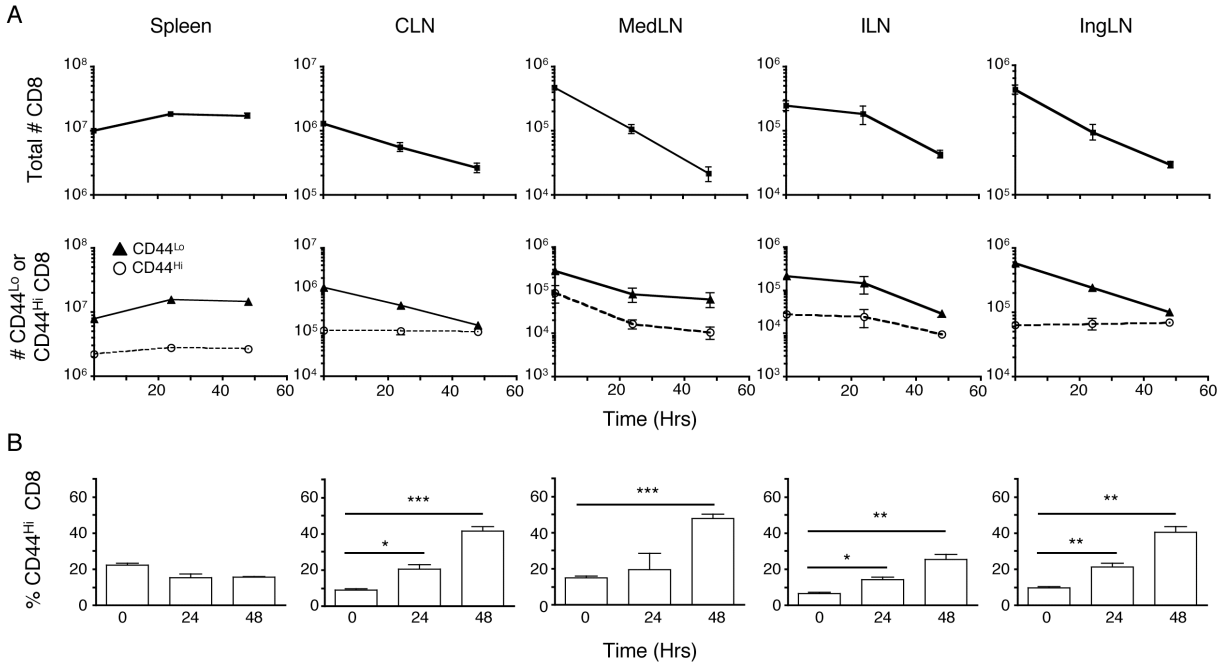


Figure 3.4. Ratio of memory T cells increases in WT mice following inhibition of L-selectin mediated entry. WT mice were given 100ug of anti-CD62L blocking antibody i.v. and single cell suspensions were prepared from isolated organs at 0, 24 or 48 hours post-treatment, and stained with CD4, CD8 and CD44 mAbs. A, Enumeration of total CD8 T cells, $CD44^{high}$ CD8 T cells, and $CD44^{low}$ CD8 T cells over 48-hour time course. B, Percentage of $CD44^{high}$ CD8 T cells at 0, 24, and 48 hours. Data was collected from n=2-6 mice for each time-point. (*$p<0.05$, ** $p<0.01$, and *** $p<0.001$)

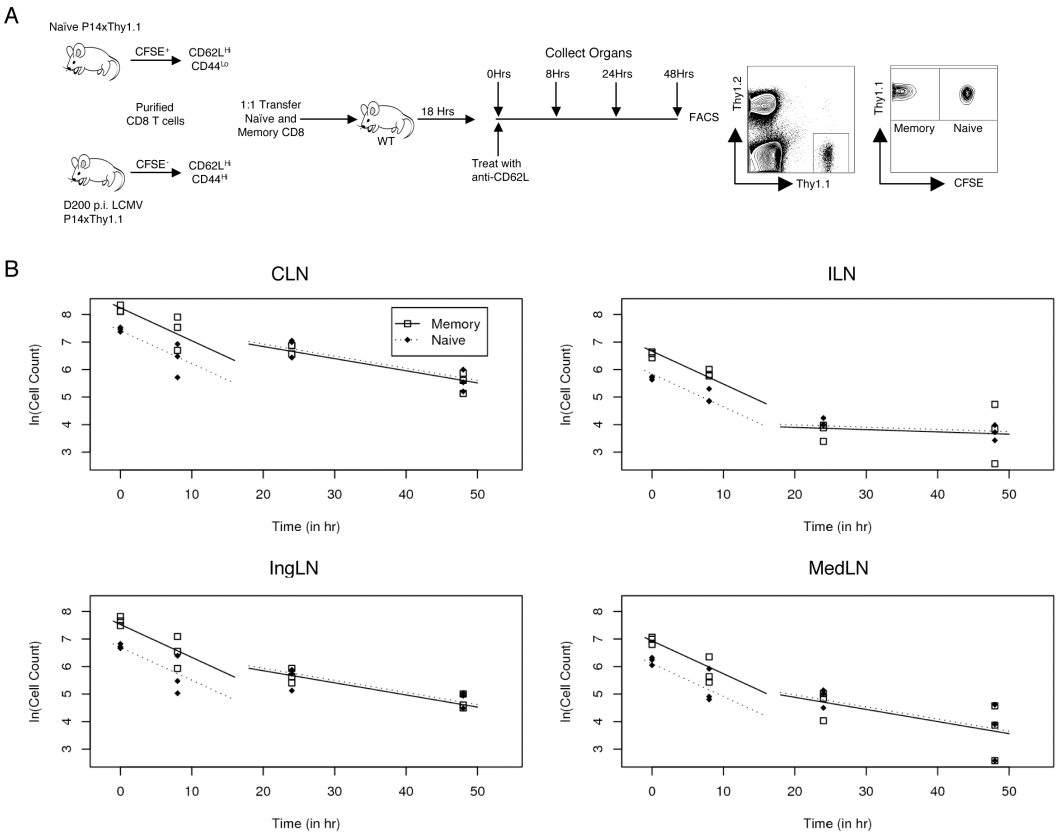


Figure 3.5. Exit rates of naïve and memory CD8 T cells from LN are similar, but residency time in the LN is density-dependent**.** CD8 T cells were purified from naïve Thy1.1xP14 Tg mice ($CFSE^+$) or immune (day 200 p.i.) Thy1.1xP14 Tg chimeric mice ($CFSE^-$), transferred into WT Thy1.2 recipient mice, and 18 hrs later mice were given 100ug anti-CD62L blocking antibody i.v. LNs were isolated 0, 8, 24, and 48 hours post treatment.. *A*, Experimental design. *B*, Naïve and memory CD8 T cells were isolated from indicated organs and egress rates determined comparing early (0 to 8 hrs) and late (24 to 48-hrs) time points when total T cell density of LNs changed significantly. Data represents n=3 mice for each time point. Open symbols show memory T cells, and filled symbols show naïve T cells.

Figure 3.6. Memory T cells are significantly impaired in trafficking to LNs of FtDKO mice. CD8 T cells were purified from naïve Thy1.1xP14 Tg mice ($CFSE^+$) or immune (day 200 p.i.) Thy1.1xP14 Tg chimeric mice ($CFSE^-$), transferred into WT or FtDKO recipient mice, and recipient mice were sacrificed 18 hours later. Thy1.1 was used to differentiate endogenous from transferred T cell populations in indicated organs. *A*, Experimental design. *B*, Pie charts showing the distribution of memory CD8 T cells in WT and FtDKO recipient mice in 9 indicated organs. The smaller pie charts show the distribution of remaining (~12%) memory CD8 T cells not in bone marrow or spleen (PBMC, liver, lung, CLN, MedLN, ILN and IngLN). *C*, Enumeration of transferred naïve and memory CD8 T cells in CLN. *D*, Representative FACS analysis of CLN and PBMC. Numbers indicate the percentage of memory ($CFSE^-$) and naïve ($CFSE^+$) Thy1.1 CD8 T cells in WT and FtDKO mice. *E*, Bar graphs showing percentage of naïve or memory $Thy1.1^+$ cells comparing WT and FtDKO mice. Data was collected from n=5 WT and n=6 FtDKO from two independent experiments. (*$p<0.05$, ** $p<0.01$, and *** $p<0.001$)

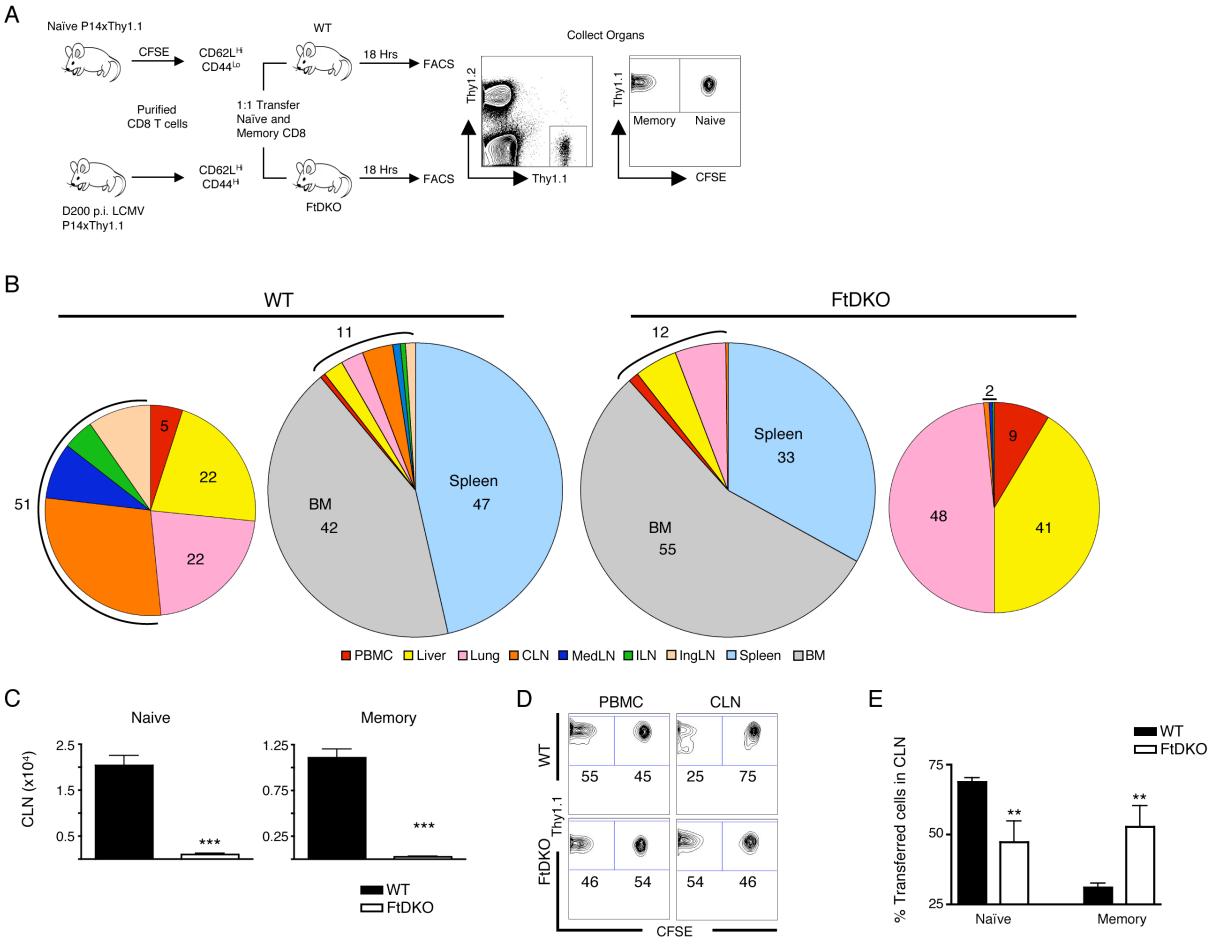
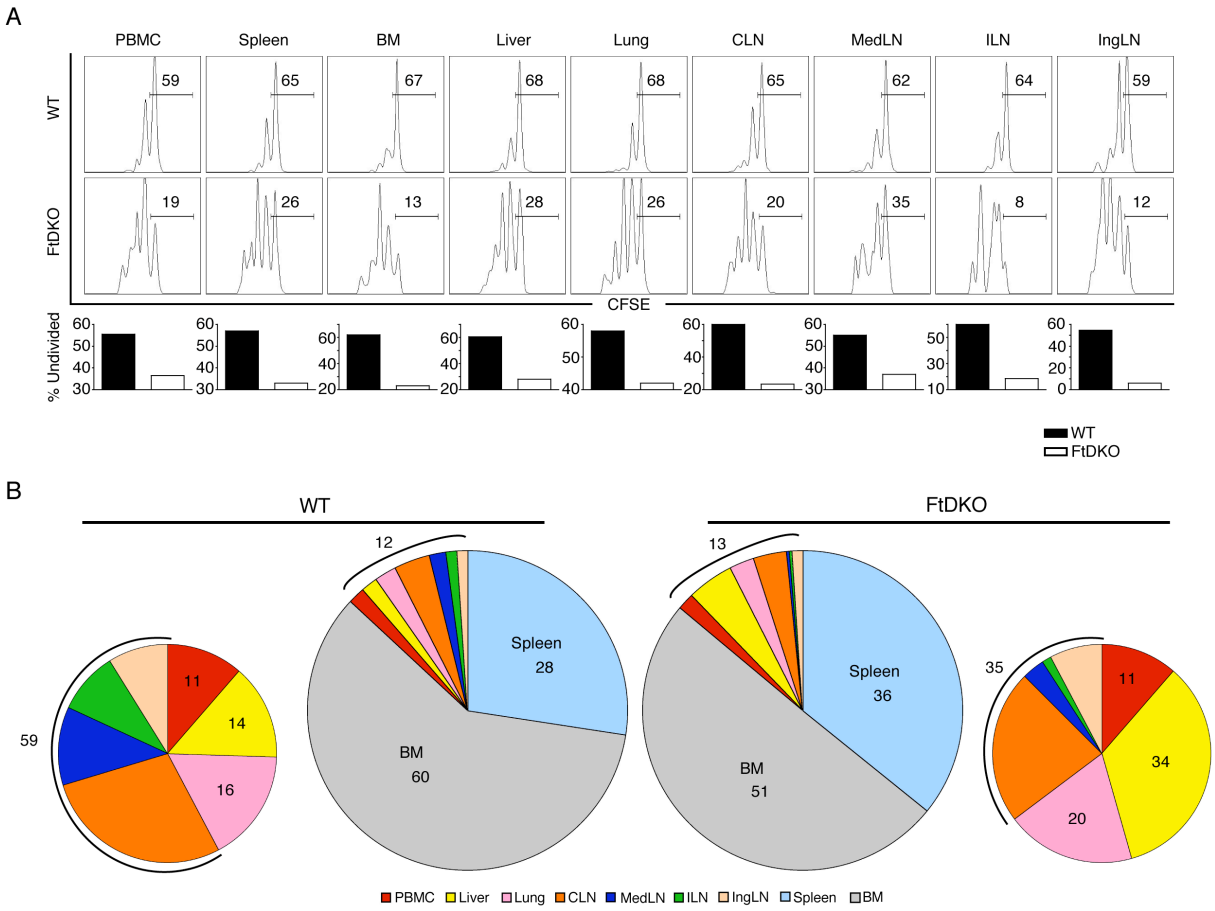

A
Naïve P14xThy1.1
CFSE
CD62L^Hi CD44^Lo
WT
18 Hrs
FACS
Collect Organs
Purified CD8 T cells
1:1 Transfer Naïve and Memory CD8
Thy1.2
Thy1.1
Thy1.1
Memory
Naïve
CFSE
CD62L^Hi CD44^Hi
18 Hrs
FACS
D200 p.i. LCMV P14xThy1.1
FtDKO
B
WT
FtDKO
11
12
2
5
22
22
51
BM 42
Spleen 47
Spleen 33
BM 55
9
48
41
PBMC Liver Lung CLN MedLN ILN IngLN Spleen BM
C
Naive
Memory
CLN (x10⁴)
2.5
1.5
0.5
1.25
0.75
0.25
***
***
WT
FtDKO
D
PBMC
CLN
WT
FtDKO
Thy1.1
55
45
25
75
46
54
54
46
CFSE
E
WT
FtDKO
% Transferred cells in CLN
75
50
25
**
**
Naïve
Memory

Figure 3.7. Memory T cells show increased homeostatic proliferation and accumulation in FtDKO mice. P14x Thy1.1^{+} CFSE^{+} memory CD8 T cells were transferred into WT or FtDKO recipient mice, and 21 days later recipient mice were sacrificed. Thy1.1^{+} CFSE^{+} memory CD8 T cells were enumerated in organs and CFSE dilution analyzed. *A*, Representative histograms showing CFSE dilution of transferred memory cells in indicated organs at 21 days. Number indicates percentage of undivided cells in each panel. Bar graphs show percentage of undivided memory CD8 T cells in WT compared to FtDKO mice. *B*, Pie charts showing distribution of memory CD8 T cells in WT and FtDKO recipient mice in 9 indicated organs at 21 days post-transfer. The smaller pie charts show the distribution of remaining memory CD8 T cells (~13%) not in bone marrow or spleen (PBMC, liver, lung, CLN, MedLN, ILN and IngLN). Data was collected from n=2 mice in each group. For the FtDKO group, one animal was Ft-VII $^{-/-}$ / Ft-IV $^{+/-}$.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MedLN
ILN
IngLN
WT
FtDKO
59
65
67
68
68
65
62
64
59
19
26
13
28
26
20
35
8
12
CFSE
% Undivided
WT
FtDKO
B
WT
FtDKO
59
11
14
16
12
Spleen
28
BM
60
13
Spleen
36
BM
51
35
11
34
20
PBMC
Liver
Lung
CLN
MedLN
ILN
IngLN
Spleen
BM

Table 3.I.

Treatment	Rate (h^{-1})	SE	*t*-value	*p*-value
Early	0.1195	0.015360	-7.782	$< 10^{-10}$
Late	0.0440	0.005912	-7.450	$< 10^{-10}$
Late ILN	0.0082	0.010240	-0.806	0.423

Table I: Estimates of T-cell egress.

Chapter 4

Conclusions

One of the fundamental properties of the adaptive immune system is the ability to direct T cells and APCs in a site-specific manner so that an effective immune response can be elicited. This interaction of T cells and APCs typically occurs in secondary lymphoid organs, where conditions are optimized for rare Ag-specific T cells to come in contact with APCs in order to become activated (Figure 4.1). Upon activation, the activated T cells can proliferate, differentiate, leave the secondary lymphoid organ, and enter the target organ to degranulate after TCR triggering and release granzyme B or perforin (CD8 T cells) or recruit additional immune cells (CD4 T cells) [146]. At the peak of the CD8 T cell response, 90% to 95% of effector T cells die leaving a pool of cells that differentiate into memory T cells that can act as sentinels for possible re-infection [146]. With an understanding of the molecular mechanisms that govern cell migration, we can begin to employ successful therapeutic strategies to ameliorate human disease caused by misguided cell migration.

Naïve T cells accumulate in lung while memory T cells accumulate in LNs

In uninfected, specific pathogen free mice, the majority of T cells are naïve T cells ($CD44^{low}$) with a smaller number of effector or memory T cells ($CD44^{high}$). Memory T cells, which are antigen experienced, can establish primary residency in secondary lymphoid organs (central memory T cells) or peripheral organs (effector memory T cells), however both subsets have the capacity to exert effector function (i.e. degranulate or help recruit additional immune cells). In WT mice, naïve T cells are

found preferentially in secondary lymphoid organs while memory T cells can be found predominantly in bone marrow and spleen. When studying the distribution of T cells in FtDKO mice, it is clear that the loss of selectin ligands has a drastic effect on T cell location. There is a severe reduction in the number of T cells that enter LNs in FtDKO mice, but naïve T cells are present in the spleen and blood. One surprising observation was that these mice demonstrate an increased numbers of naïve T cells in the lung. To initially investigate the reason for increased naïve T cells in the lung, we performed an analysis of lung sections and found no evidence of iBALT. In contrast, CCR7 KO mice, which also show defective migration to LNs, show evidence of iBALT [89]. Additionally, we made another observation in uninfected FtDKO mice where we observed that the frequency of$CD44^{high}$ memory T cells was higher in the LNs compared to WT. These results raised a few questions that we wanted to investigate. Why were we observing more naïve T cells in the lung of FtDKO mice? Why were there greater frequencies of "memory" $CD44^{high}$ T cells in the LNs of uninfected FtDKO mice?

Generation of effector and memory T cells and adoptive transfer

To address these questions, we assayed the distribution of T cells during a viral response using the LCMV-Armstrong model. In the mouse, the number of Ag-specific naïve T cells for a specific epitope is quite low (100-200 epitope specific cells), while the number of Ag-specific memory T cells is significantly higher in an immune host (500,000 epitope specific cells) [149]. However, both T cell populations require interaction with

APCs for activation and this typically occurs in LNs and other secondary lymphoid organs. Upon interaction with TCR displaying cognate antigen, naïve T cells become activated and initiate proliferation. Following proliferation, the newly differentiated effector T cells begin to secrete effector cytokines (i.e. IFNg) and or display killer function. In contrast, memory T cells can immediately secrete effector cytokines and or display killer functions following TCR engagement and also begin to proliferate [150]. In addition to differences in activation and early effector function, naïve and memory T cells preferentially localize in different sites. It has been shown that naïve T cells primarily migrate between secondary lymphoid organs because of high expression of L-selectin. On the other hand, memory T cells may or may not express L-selectin and also express surface receptors that facilitate entry to non-lymphoid organs such as the skin via the skin homing molecule CLA [6].

In our experiments, Ag-specific T cells were activated and expanded in FtDKO mice, however T cell numbers were reduced in LNs and lung compared to WT mice at day 8 post infection. This result corroborates other studies suggesting that the upregulation of fucosyltransferase –IV and –VII in activated T cells is important to access the inflamed lung [90]. After the infection was cleared and memory established, we found that total T cell numbers in the FtDKO lung were still reduced. However, Ag-specific memory CD8 T cell numbers in the LNs were similar to WT. This suggested that unlike naïve CD8 T cells and Ag-specific effector CD8 T cells, Ag-specific memory CD8 T cells potentially showed no defects in either entering the LNs, or were not exiting the LNs as rapidly as naïve T cells (i.e. they were being preferentially retained).

To determine whether naïve T cells homed to the lungs of FtDKO, we performed an adoptive transfer of naïve and central memory CD8 T cells. By transferring a similar ratio of *bona fide* Ag-specific naïve and Ag-specific central memory CD8 T cells of the same peptide specificity into recipient WT and FtDKO mice, we could directly test the distribution of naïve versus memory T cell populations. In the lungs of FtDKO recipient mice, we found that transfer of both naïve and central memory T cell populations resulted in greater numbers in the lung when T cells could not enter LNs. If we had performed an additional experiment using effector memory T cells, we predict that effector memory T cells would preferentially accumulate in the lung as well, since effector memory T cells preferentially home to peripheral organs [6]. To further understand the observation of increased naïve T cells in the lung, we performed additional transfer experiments in WT mice where we blocked LN access by either treating CD8 T cells with a Gαi signal disruptor (PTx) or treating recipient mice with anti-CD62L antibody. These experiments resulted in more T cells in the blood and more T cells in non-lymphoid organs, like the lung. Performing the converse experiment of disrupting T cell exit from the lymph node with the S1P1 agonist, FTY720 resulted in fewer cells in the blood and non-lymphoid organs. These data supported our hypothesis that if entry to LNs is disrupted, T cells will re-distribute and accumulate in non-lymphoid organs (Figure 4.2).

Entry to LN and exit from LN is similar in both naïve and memory T cells

As mentioned previously, Ag-specific memory CD8 T cells were enriched in the LNs of FtDKO mice. To determine whether memory T cells entered LNs via selectin ligand independent mechanisms, we transferred WT naïve and central memory T cells into WT and FtDKO mice. We found that both subsets were significantly reduced in the LNs of FtDKO mice, implying that both naïve and memory T cells require selectin-selectin ligand interactions to optimally enter LNs. If we had performed an additional experiment, by isolating naïve and memory T cells from FtDKO mice, which cannot express selectin-ligands, and transferring them into WT or FtDKO recipient mice, we might observe different results. Naïve T cells which do not express fucosyltransferase –IV and –VII would likely not have altered trafficking patterns in recipient WT mice because the environment in the WT mouse would be similar. However, transferred memory T cells into WT recipients would likely be affected by the absence of E- and P-selectin ligands on the transferred T cell and as a consequence may demonstrate reduced trafficking to peripheral organs.

Since memory CD8 T cells were not entering the LNs using selectin ligand independent mechanisms, these data suggested that naïve and memory CD8 T cells might have different retention rates within the LNs. To determine the possibility of different exit rates, we transferred naïve and central memory CD8 T cells into recipient WT mice and treated the recipient mice with anti-CD62L to prevent new cells from entering the LN. We observed no statistical differences in naïve versus memory T cell

egress. However, in a recent report, the data suggested that naïve CD4 and memory CD4 T cells have different retention rates, possibly due to CD4 T cell interaction with MHC-II and CD8 T cell interaction with MHC-I [48]. In our study, we found that both populations of CD8 T cells exited similarly, but the rate of their exit was dependent on the density of cells in the LN. As T cell density decreases, T cells show longer retention in LNs.

These data prompted us to hypothesize that memory cells that do move through the low density LN may undergo increased homeostatic proliferation. Many studies have shown that T cells can proliferate via emptiness induced homeostatic proliferation as well as Ag-driven proliferation [147,148]. In emptiness induced homeostatic proliferation, it has been found that as naïve CD8 T cells divide, they begin to express memory T cell markers such as CD44 and CD122 [147]. Of note, the cells that underwent emptiness induced homeostatic proliferation were capable of producing IFNg, but the levels of IFNg were not as high as the levels produced by T cells that underwent Ag-driven proliferation [147]. To determine whether T cells showed increased homeostatic proliferation in FtDKO mice, we adoptively transferred CFSE labeled memory T cells into FtDKO mice and assayed proliferation several weeks later. We found increased proliferation of memory T cells in FtDKO mice compared to WT. These data suggested that although migration of memory T cells into selectin ligand deficient LNs is greatly impaired, the small number of memory T cells that do enter, will undergo substantial proliferation, and subsequently we observe an increase in the frequency of the memory T cells in the LNs (Figure 4.3). However, one other important

factor to consider is that naïve T cells likely undergo increased proliferation in FtDKO mice as well and that the increased frequency of $CD44^{high}$ "effector/memory" cells that we observed in the LNs of uninfected FtDKO mice might also be due to this emptiness induced proliferation [147].

FtDKO as tools for studying T cell trafficking

By using FtDKO mice, we were able to perform elegant experiments that allowed us to identify similarities and tease apart differences between naïve and memory T cell trafficking. We discovered that defects in T cell trafficking to LNs results in accumulation of naïve T cells in non-lymphoid organs of FtDKO mice and also that memory T cells accumulate in LNs due to increased homeostatic proliferation. These two previously unknown phenotypes in FtDKO mice revealed that naïve and memory T cells display unique mechanisms for compensating for selectin ligand deficiency. Using these mice as a tool to understand the impact of altered trafficking may help treatments for immune cell trafficking related diseases become more readily available.

Appendix

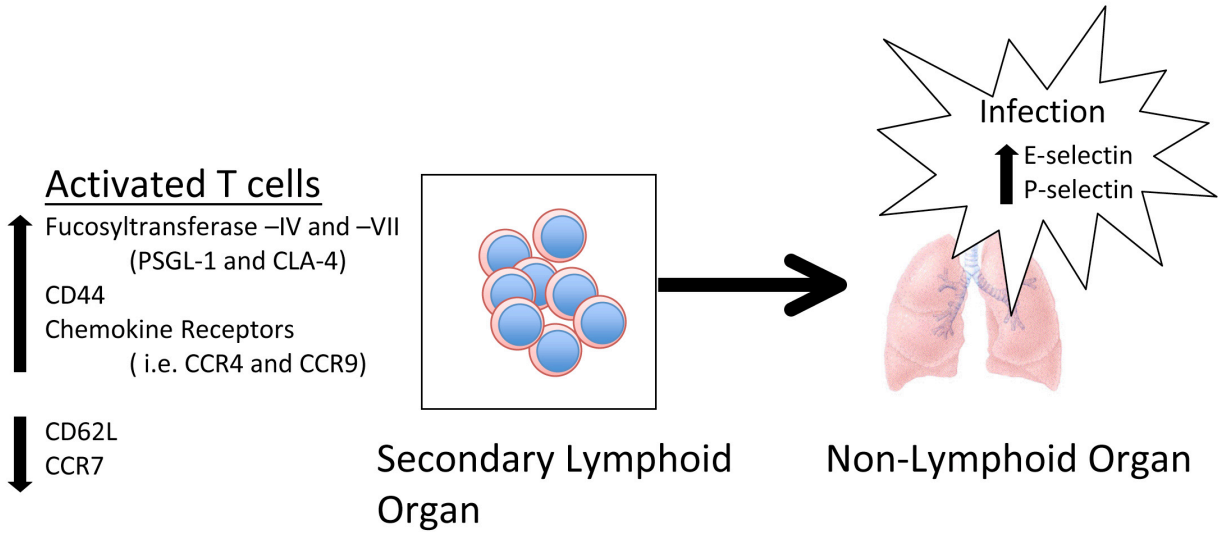


Figure 4.1. Effector T cell trafficking. Upon infection, cytokines will induce the upregulation of E- and P-selectin on epithelial cells. APCs from the site of infection will migrate towards the draining lymph node and interact with T cells. When the antigen specific T cell recognizes its cognate antigen, it will begin to upregulate fucosyltransferase –IV and –VII (to modify glycoproteins, PSGL-1 or CLA-4), CD44, and various chemokine receptors (i.e. CCR4 and CCR9), downregulate CD62L and CCR7 and proliferate. Activated T cells can then migrate back to the site of infection in order to clear antigen.

Figure 4.2. Naïve T cell trafficking patterns. A) Naïve T cells primarily enter LNs from the blood (red arrow) where they can engage adhesion molecules on the HEV. After transiting through the LN environment, naïve T cells will enter the efferent lymphatics (green arrow), enter the thoracic duct, and finally move back into blood circulation. Once in the blood, naïve T cells can transit through non-lymphoid organs (i.e. lung). Some naïve T cells will bypass HEVs and continue to travel though the blood to briefly reside in non-lymphoid organs. B) If entry to LN is blocked (selectin ligand deficiencies - FtDKO, Gαi signaling –PTx, or anti-L-selectin antibody), a greater number of naïve T cells will remain in the blood and enter non-lymphoid organs. C) If exit from LN is blocked (S1P1 agonist – FTY720), naïve T cells will accumulate in the LNs and as a consequence reduce the number of naïve T cells in the blood and non-lymphoid organs.

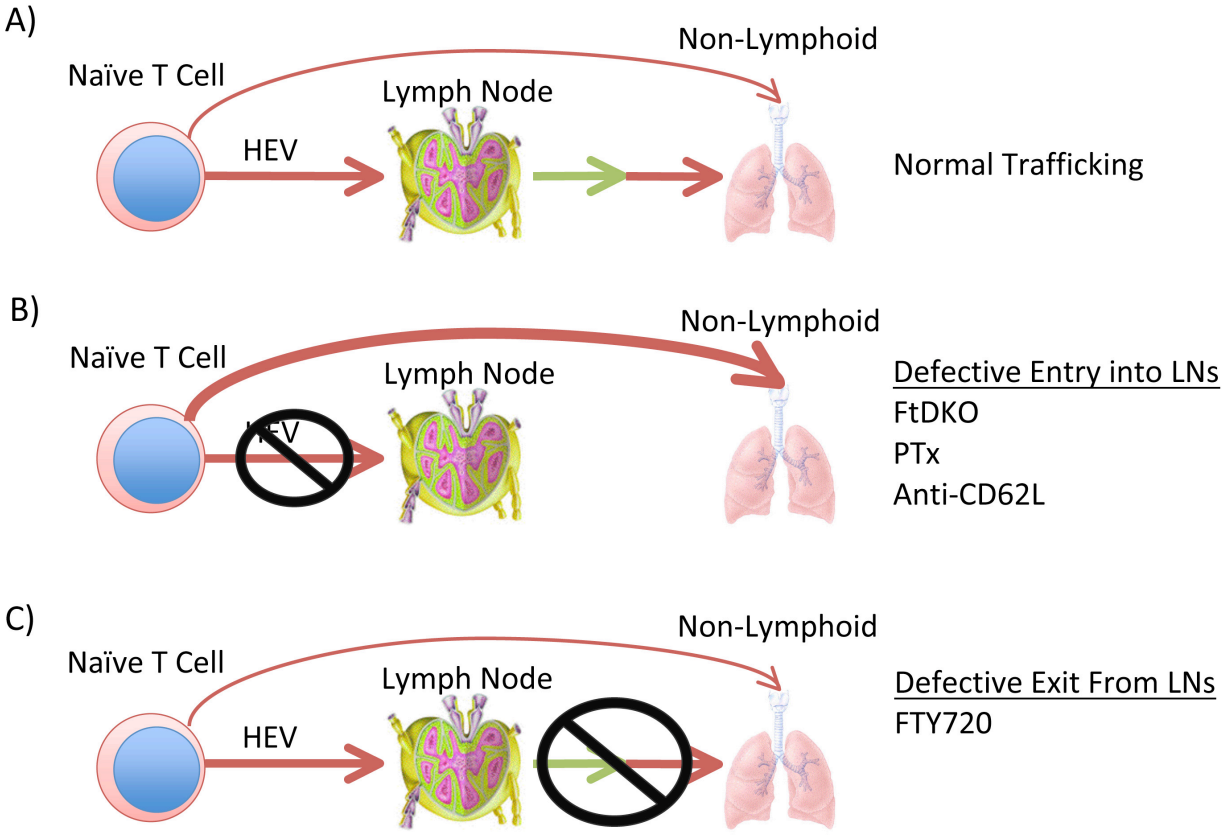
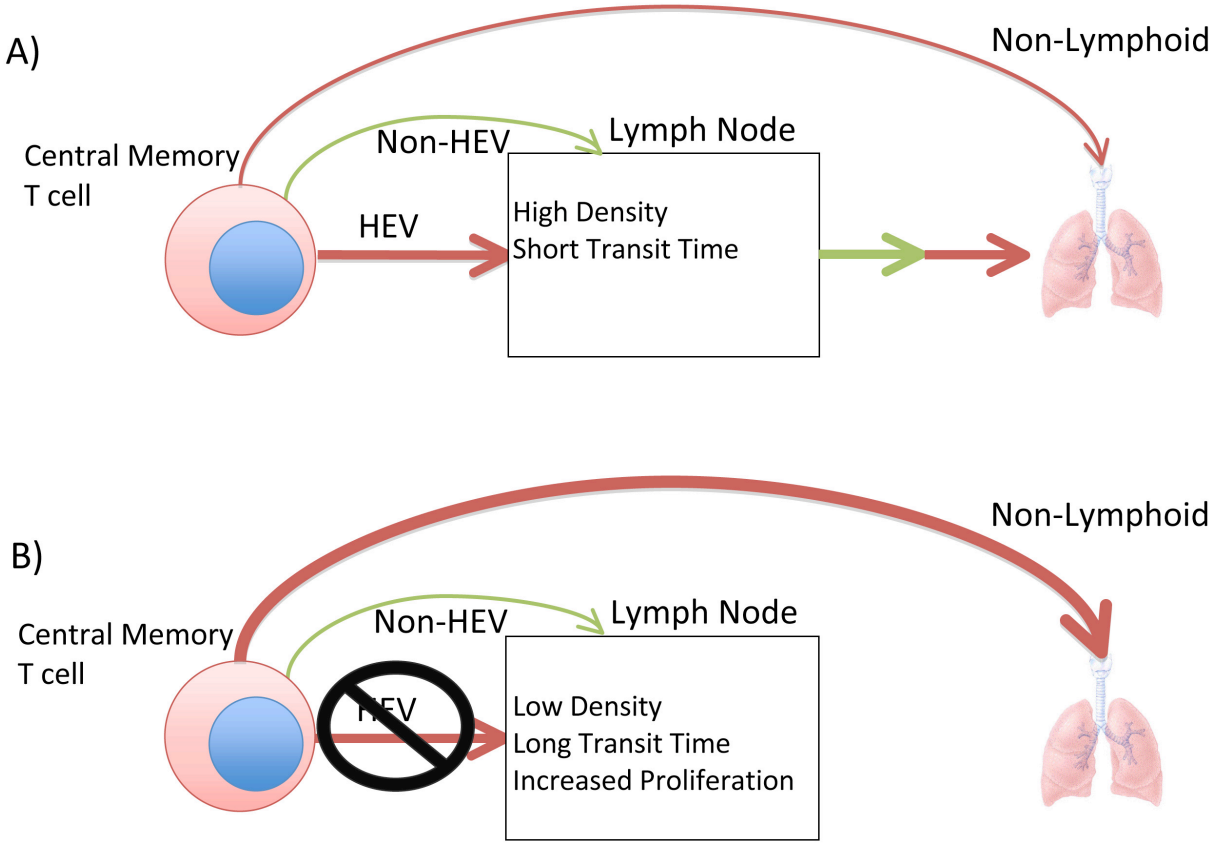

A)
Naïve T Cell
Non-Lymphoid
Lymph Node
HEV
Normal Trafficking
B)
Naïve T Cell
Non-Lymphoid
Lymph Node
HEV
Defective Entry into LNs
FtDKO
PTx
Anti-CD62L
C)
Naïve T Cell
Non-Lymphoid
Lymph Node
HEV
Defective Exit From LNs
FTY720

Figure 4.3. Central memory T cell trafficking patterns. A) Central memory T cells preferentially home to secondary lymphoid organs (i.e. lymph node) via the blood (red arrow) where they can engage adhesion molecules on the HEV. A small number of central memory T cells can also enter lymph nodes through the afferent lymphatics (non-HEV) route. The central memory T cells can then migrate through the LN (short duration) and enter into the efferent lymphatics (green arrow), enter the thoracic duct, and finally move back into blood circulation. Once in the blood, central memory T cells can transit through non-lymphoid organs (i.e. lung). Some central memory T cells will bypass HEVs and continue to travel though the blood to briefly reside in non-lymphoid organs. B) If entry to lymph node is blocked (selectin ligand deficiency – FtDKO or anti-L-selectin antibody), a greater number of naïve T cells will remain in the blood and enter non-lymphoid organs. The small number of cells that do enter the LNs via the afferent lymphatics, will experience a longer than normal residency time, due to decreased density, where they will undergo increased proliferation.


A)
Non-Lymphoid
Central Memory
T cell
Non-HEV
Lymph Node
HEV
High Density
Short Transit Time
B)
Non-Lymphoid
Central Memory
T cell
Non-HEV
Lymph Node
HEV
Low Density
Long Transit Time
Increased Proliferation

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Vita

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