THE ROLE OF THE G-PROTEIN COUPLED RECEPTOR C-C CHEMOKINE RECEPTOR 7 IN T LYMPHOCYTE MIGRATION AND BREAST CANCER METASTASIS

By

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LIST OF ABBREVIATIONS

β1- Beta 1 integrin subunit

Bcl-2 – B Cell Lymphoma 2

BMDC – Bone Marrow Derived Dendritic Cells

CD62L – L selectin

CCR7 - C-C Chemokine Receptor 7

CCL19 - Mip3β, ELC, CKb11, exodus 3

CCL21 - SLC, 6Ckine, TCA4, exodus 2

CFSE - (5uM) 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

CMTMR - (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)

Edg-1 - Endothelial Differentiation Gene 1 (mouse $S1P_1$)

ERK1/2 -Extracellular Signal-Related Kinases 1 and 2 (P44/42)

ERK5 – Extracellular Signal-Related Kinase 5

FITC - Flourescein isothiocyanate

GDP – Guanosine Diphosphate

GPCR - G-protein Coupled Receptor

GRK – G-protein coupled receptor kinase

GTP – Guanosine Triphosphate

HEV – High Endothelial Venules

JAK-2 – Janus Kinase 2

KLF2- Kruppel Like Factor 2

MKP-3 – MAP Kinase Phosphatase-3, DUSP6

mM - Millimolar

nM – Nanomolar

PAC-1 – Phosphatase of Activated Cells-1

PI3K – Phosphatidylinositol 3-Kinase

PLCγ1 - Phospholipase C gamma 1

PTX - Pertussis Toxin

PyVmT - Polyoma virus middle T antigen

S1P – Sphingosine 1 Phosphate (Ligand for $S1P_1$ or EDG-1)

$S1P_1$ - Sphingosine 1 Phosphate Receptor (human – Edg-1)

TNFα – Tumor Necrosis Factor α

uM - Micromolar

VCAM-1 – Vascular Cell Adhesion Molecule-1

CHAPTER 1 – SIGNIFICANCE AND AIMS

1.1 Significance

Cell migration is an intricate process that is important during immune responses and cancer metastasis. Immune cells must be able to migrate throughout the body in order to properly mount effective immune responses. Similarly, a successful metastatic cancer cell must also be able to migrate from the primary tumor to a secondary site. Cell migration is carried out via a process called chemotaxis, in which cells sense gradients of chemoattractants, through their chemotactic receptors. Activation of chemotactic receptors leads to downstream signaling processes required for integrin-mediated migration. Integrins are adhesion proteins that allow the cell to adhere to surfaces, propel itself along these surfaces and de-adhere so that the cycle can be repeated. Both immune cells and cancer cells use specific chemotactic receptors to signal to integrins in order to mediate migration to precise locations that express specific gradients of chemokines.

Under normal conditions immune cells constantly migrate throughout the body scanning lymphoid organs and other tissues looking for foreign invaders, such as bacteria or viruses. Immune cells use chemotactic receptors to migrate into specific areas of the body such as the bone marrow, lymph nodes, lungs and brain. T lymphocytes are major players in the immune response as they are important for activating B lymphocytes, secreting cytokines that further amplify the immune response, retaining memory that allow for a quicker immune response and, in certain cases being cytotoxic. The chemokine CCL21 is highly expressed in high endothelial

venules (HEVs), and through unknown signaling mechanisms, directs naïve T lymphocytes that express C-C Chemokine Receptor 7 (CCR7) into lymph nodes from the blood through HEVs. Inside lymph nodes, the chemokines CCL19 and CCL21 direct naïve T lymphocytes that express CCR7 into regions of the lymph node where they can interact with other cells such as B lymphocytes and dendritic cells. In the lymph node naïve T lymphocytes scan dendritic cells that present an antigen, broken down bacterial or viral peptides, which the naïve T lymphocyte can recognize. Dendritic cells also express the chemokine CCL19 which attract naïve T lymphocytes to their vicinity. If a naïve T lymphocyte fails to recognize antigen presented by a dendritic cell, a mechanism is in place that will allow the naïve T lymphocyte to exit the lymph node and to migrate to other lymph nodes where the cells can continue to look for antigen it recognizes. However, if a T lymphocyte recognizes antigen presented by the dendritic cell, the naïve T lymphocyte becomes activated, differentiates, exits the lymph nodes and migrates into the periphery to participate in the inflammatory response. It is during an extended period of time of T lymphocyte/dendritic cell interactions that exit mechanisms are up-regulated, yet it is unclear what specific signal(s) is/are required to turn on this machinery and promote egress from the lymph node. During over-exaggerated allergic reactions and autoimmune disorders, T lymphocytes recruited to areas of inflammation can cause severe damage and even death. Therefore, it is important to be able to regulate the migration of naïve T lymphocytes before or while they are in lymph nodes, before they have the opportunity to exit lymph nodes and move into areas of inflammation as activated effector T lymphocytes and create further destruction of tissues.

Many types of metastatic cancers, such as breast cancer, exploit the same molecular mechanisms used by lymphocytes to become motile and migrate into specific organs. CCR7 is

up-regulated in metastatic breast cancers and has been correlated with metastasis to lymph nodes. Because CCR7's role in lymphocyte migration is to direct cells into the lymph nodes, there have only been strong correlations but no observations *in vivo* that demonstrate that CCR7 mediates this same process in breast cancer cells. Furthermore, at present it is unclear what CCR7 mediated mechanisms might control lymph node metastasis. Taken together, CCR7 represents a primary target that can potentially be used to regulate autoimmunity and breast cancer metastasis. The results of my dissertation project have increased our knowledge of the molecular mechanisms that contribute to CCR7 mediated migration through its ligands CCL19 and CCL21 in naïve T lymphocytes and development of a mouse model to study CCR7 mediated metastasis of breast cancer to the lymph nodes outlined in the three following chapters:

Chapter 3) CCR7/CCL21 Migration is Mediated by PLCγ1 and ERK1/2 in Primary T Lymphocytes.

Chapter 4) CCR7/CCL19 Mediates T Lymphocyte Expression of EDG-1.

Chapter 5) Expression of a Chemokine Receptor, CCR7, Mediates Metastasis of Breast Cancer to the Lymph Nodes and Reduces Metastasis to the Lungs in mice.

CHAPTER 2 – BACKGROUND

2.1 Cellular Migration

Cell migration is a multifaceted process that requires activation followed by de-activation of adhesive contacts, polarization of the appropriate signaling molecules and a chemokine/growth factor gradient of which to migrate towards [1]. As cells migrate along the extracellular matrix they use adhesive contacts called integrins. Integrins are unique receptors that can be activated by "inside out" signaling which occurs through activation of another cell surface receptor, such as a chemokine receptor, or "outside in" signaling which occurs via a ligand binding to the integrin itself [2]. Integrins are heterodimeric proteins that are made up of an α and β subunit. During integrin activation these subunits are typically in an extended conformation, while during de-activation the subunits return to a bent/folded confirmation [3]. When a cell receives a signal to move, integrins are rapidly polarized to the leading edge of the cell where they bind the extracellular matrix [4, 5]. Force is then generated through interactions with the actin cytoskeleton and the cell is then propelled over the extracellular matrix [6]. The integrins then release from the extracellular matrix and are recycled back into the cell to repeat the cycle again [7].

As described in Chapter 1, naïve T lymphocytes must be able to enter into and migrate within lymph nodes in order to encounter antigen presenting dendritic cells and they rely greatly on β1 integrins to accomplish this task [8]. β1 integrins bind to collagen I and IV, Vascular Cell Adhesion Molecule-1 (VCAM-1), laminin and fibronectin. Both laminin and fibronectin are expressed in high endothelial venules, the point of entry for lymphocytes migrating into lymph

nodes [9, 10]. Chemokine activation results in β1 integrin polarization at the leading edge of migrating cells and increased activation of β1 integrins [4, 8]. In human naïve T lymphocytes activation of CCR7 by its ligand CCL21 results in firm adhesion, tethering and rolling on VCAM-1, a β1 integrin ligand. [11, 12]. However, the molecular mechanisms of how CCR7 signals to β1 integrins to induce migration to lymph nodes remain unclear.

2.2 Organization and Function of the Lymph Nodes

Lymph nodes are small bean shaped structures linked together by lymphatic vessels that are found throughout the entire body. Regardless of size, lymph nodes are one of the most important sites for cellular interaction and activation of immune responses. The lymph node is divided into two individual regions including the cortex and the medulla [13] (Figure 1). The cortex can be further divided into two regions including the paracortex which is also known as the T cell area and the node cortex that includes the B cell area [14]. The paracortex (T cell area) is the location where T lymphocytes and dendritic cells interact and where lymphocytes enter into lymph nodes through high endothelial venules, a rich source of CCL21 [15]. The paracortex is populated with fibroblastic recticular cells, which are a source of CCL19 and CCL21 [16]. As lymph nodes are sites of continuous cell chemotaxis they therefore express many types of extracellular matrix molecules that assist in cell migration to different compartments including collagen, laminin, vitronectin and fibronectin [17, 18].

Lymph nodes are central sites of antigen presentation, cell to cell interactions and lymphocyte activation. Dendritic cells located in the periphery take up antigen, undergo

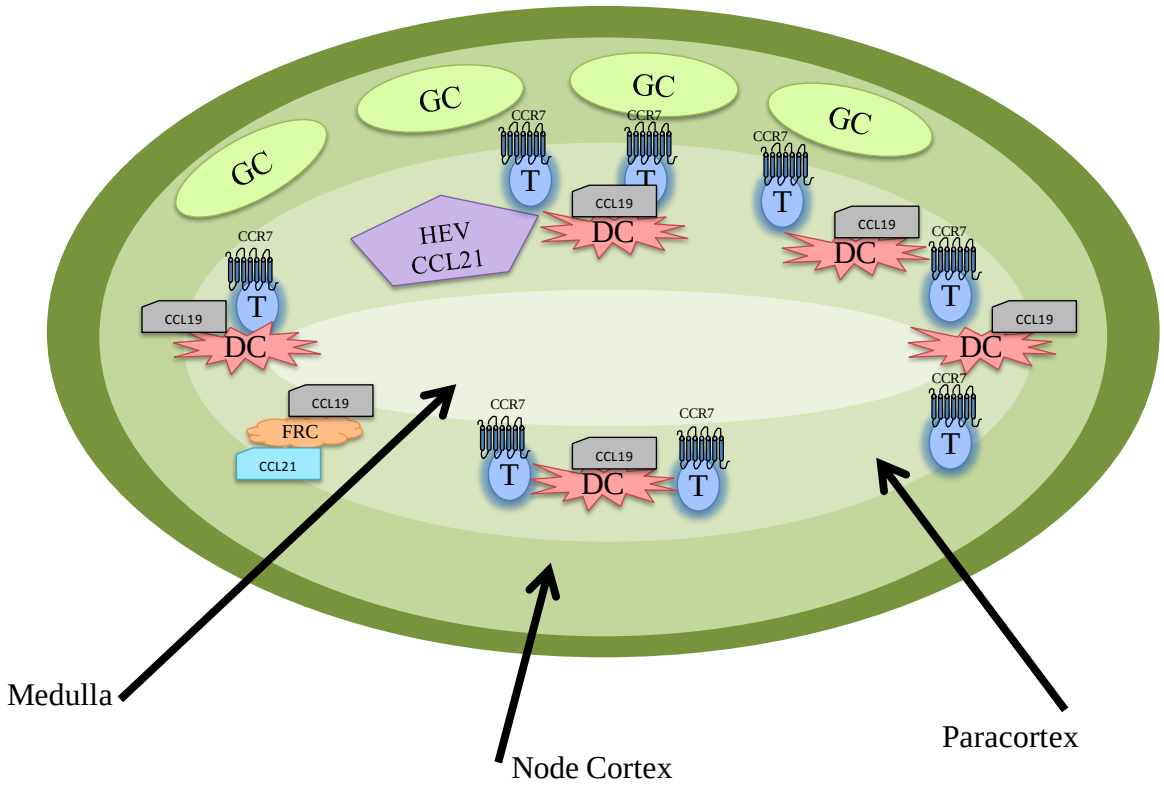

Figure 1. Organization of the lymph node. Lymph nodes are important sites of cellular interaction and activation of immune responses. The lymph node is divided into two regions, the cortex and the medulla. The cortex is further divided into the paracortex and node cortex. The paracortex includes the T lymphocyte area where high endothelial venules (HEV) are located. High endothelial venules are the entry point for T lymphocytes and express CCL21. In the paracortex T lymphocytes (T) expressing CCR7 interact with dendritic cells (DC) that express CCL19. The paracortex is also populated with fibroblastic recticular cells (FRC) expressing CCL19 and CCL21. The cortex is the B lymphocyte area and under inflammatory conditions forms germinal centers (GC), which are composed of areas of activated B lymphocytes.

maturation and migrate to regional lymph nodes, where they will display antigen for recognition by naïve T lymphocytes. Naïve T lymphocytes migrate toward lymph nodes, entering into the lymph nodes through high endothelial venules where they first encounter CCL21. Upon entering the lymph nodes naïve T lymphocytes immediately begin to scan dendritic cells that express CCL19 [19], which facilitates interactions between the two cell types. In the absence of antigen naïve T lymphocytes and dendritic cells can make upwards of 5,000 contacts per hour, which is amplified during an immune response [20, 21]. In the presence of antigen, naïve T lymphocytes during early stages of interaction with dendritic cells, will make transient contacts that last for minutes, with little activation detected among the T lymphocytes [22]. Following these brief interactions, naïve T lymphocyte will form stable interactions with dendritic cells that can last up to 36-48 hours [22]. During this stable interaction the T lymphocyte becomes activated, proliferates and eventually detaches from the dendritic cell [23]. Lastly, the activated T lymphocytes exit the lymph nodes and return to the circulation to carry out their effector functions in the periphery.

2.3 C-C Chemokine Receptor 7 (CCR7) and its Ligands CCL19 and CCL21

CCR7 is expressed by naïve T lymphocytes, mature dendritic cells (DC), natural killer cells (NK), central memory T cells and T regulatory cells (Tregs) [24]. CCR7 binds two chemokine ligands, CCL19 and CCL21. CCL19 is expressed in the thymus, spleen, stromal cells of the lymph nodes, and dendritic cells [16, 25, 26]. CCL21 is expressed in the appendix, spleen, high endothelial venules and stromal cells of the lymph nodes [16, 27, 28]. To date the major role of CCR7 in immunity is to direct T lymphocyte migration into secondary lymphoid organs

such as the lymph nodes. The importance of this function lies in the positioning of cells within the lymph node such that activation of effector cell subsets like T and B lymphocytes occurs. If T lymphocytes fail to come into contact with antigen presenting dendritic cells either because the dendritic cells can't migrate to the lymph node or T lymphocytes can't find the dendritic cells in the lymph node, then T lymphocytes simply do not become activated. In addition, if T lymphocytes are unable to find and activate B lymphocytes, then the immune response is diminished. In support of this function, mice lacking CCR7 have severely reduced numbers of CD4+ T cells in lymph nodes while increased numbers of these cells are found in the blood and bone marrow [29]. In addition, there is structural rearrangement of lymph nodes, such as B cell follicles in the paracortex, which are normally located in the cortex and lymph nodes are also devoid of T cell areas normally located in the paracortex [29]. CCR7 knockout mice also have impaired T cell and B cell activation and diminished lymphocyte responses during immune challenge [29].

In mice CCL21 is expressed in two forms that differ at nucleotide position 65, resulting in one that encodes a serine and the other a leucine [30]. The leucine form is expressed in non-lymphoid tissues while the serine form is expressed in lymphoid tissues and resembles the expression pattern of human CCL21 [30]. Paucity of lymph node T cells (*plt*) is a spontaneous mutant in mice that results in the deletion of both CCL21 (serine form expressed in lymphoid tissue) and CCL19 [16, 30]. Similar to the CCR7 knock out mouse, *plt* mice display a loss of T cells and DCs in the lymph nodes, spleen and Peyer's patches and as expected *plt* are more susceptible to viral infections [31]. Recent studies have demonstrated a role for CCL19 and CCL21 in the development of lymph nodes, Peyer's patches and spleen [32, 33]. However, studies from the *plt* mouse make it difficult to delineate specific roles for each ligand.

The development of a CCL19 knock out mouse and CCL19 (8-83) and CCL21 (mSLC4) antagonists have provided more insight into the role for these ligands. A study carried out in a CCL19 knock out mouse revealed that CCL19 was not essential for development of lymphoid tissue and lack of CCL19 did not affect the ability of naïve T cells to migrate into secondary lymphoid organs, suggesting that CCL21 may be more important for these functions [34]. In support of these studies, it has been shown that ectopic expression of CCL21 alone is sufficient for development of lymphoid tissue in pancreatic islets [35]. Antagonists developed against CCL19 (8-83) and CCL21 (mSLC4) also suggest more important functions. Similar to the CCL19 knock out mouse, the CCL19 antagonist 8-83 does not affect the ability of lymphocytes to enter into lymph nodes, however lymphocytes accumulate in peripheral and mesenteric lymph nodes during an immune response, suggesting that CCL19 might play a role in lymphocyte egress from lymphoid tissues [36]. In support of these studies, use of the CCL21 antagonist (mSLC4) determined that cells failed to migrate efficiently into secondary lymphoid organs [37]. Although a clear predominate role has emerged for CCL21 in mediating migration into lymph nodes and a possible role for CCL19 mediating egress has been suggested, the precise molecular mechanisms of these process are not understood.

2.4 G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are involved in many everyday human sensations. For example, they are the detection molecules for light, odor and taste as well as hormones, neurotransmitters and chemokines. GPCRs play a role in many important physiological processes including neurological, cardiovascular, endocrine and immunity [38]. Therefore, it is

not surprising GPCRs are a major target for drug discovery and that 60% of all drugs available today target GPCRs[39].

GPCRs are large transmembrane receptors that span the membrane seven times and couple to G-proteins to transmit signals into the cell. When a G-protein coupled receptor binds to a ligand the receptor undergoes a conformational change that results in coupling to a G protein [40]. G proteins are heterotrimeric molecules made up of α, β and γ subunits. Receptor activation results in an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit of the G protein. The Gα-GTP bound subunit then dissociates from the Gβγ subunit [41]. Once dissociation occurs each subunit then can initiate downstream signaling events. The Gα subunit family is composed of Gαs, Gαi, Gαq and Gα12/13 and is responsible for regulating activity of adenylyl cyclase, calcium channels, tyrosine kinases, mitogen protein activated kinase (MAPK), and phospholipase C (PLC) [42]. Gβγ subunits can also activate a variety of signaling molecules including G-protein receptor kinases (GRKs), phosphatidylinositol 3-kinase (PI3K), MAPK and PLC [42]. Activation of the Gα and Gβγ pathways leads to cellular events such as proliferation, differentiation and migration.

2.5 Entrance into Lymph Nodes: Signaling Through the CCR7 Receptor in T Lymphocytes

As previously mentioned G-protein coupled receptor activation of downstream signaling events requires coupling to a G-protein. Pertussis toxin specifically targets the Gαi family of G-proteins and adenosine diphosphate (ADP) ribosylates the Gαi subunit resulting in the inability of the G-protein to interact or couple with the receptor [43]. Studies using pertussis toxin have demonstrated that CCR7 couples to the Gαi G-protein and is required for migration to CCL21

and CCL19 [44, 45]. Additionally, CCR7 mutants that lack the C-terminal tail of the receptor also are unable to activate the Gαi subunit resulting in a loss of extracellular signal-related kinase 1and 2 (ERK1/2) activation and migration [46].

Following GPCR activation and release of Gα/Gβγ subunits, CCR7 becomes phosphorylated at its C terminus, which allows for binding of arrestins (commonly referred to as β-arrestin 1 or β-arrestin 2). Arrestins are important for receptor internalization, migration and act as adaptor proteins that mediate signaling through multiple proteins such as MAPKs [47] (Figure 2). Interestingly, β-arrestin 1 or β-arrestin2 are not required for CCR7 mediated migration in response to CCL21 activation [48]. In contrast, both β-arrestin 1 and β-arrestin 2 are required for migration to CCL19 [48]. Additionally, depletion of β-arrestin 2 lead to a loss of ERK1/2 phosphorylation in HEK293 cells stimulated with CCL19 [49].

Mitogen Activated Protein Kinase (MAPK) signaling is required for many cellular functions such as differentiation, survival and migration. G-protein coupled receptors are well known for their ability to activate MAPK signaling cascades [50-52]. Stimulation of CCR7 with either CCL19 or CCL21 results in phosphorylation of the MAPK, ERK1/2 in HEK293 cell lines [49]. In primary T and B lymphocytes ERK1/2 is rapidly and transiently phosphorylated in response to CCR7 stimulation by CCL21 [53, 54]. Rapid and transient phosphorylation of ERK1/2 in aortic smooth muscle cells is necessary for migration [55], suggesting that transient phosphorylation of ERK1/2 might also be required to induce migration of naïve T lymphocytes. However, to date it remains unclear if transient phosphorylation of ERK1/2 is required for CCR7 mediated migration to CCL21 or CCL19.

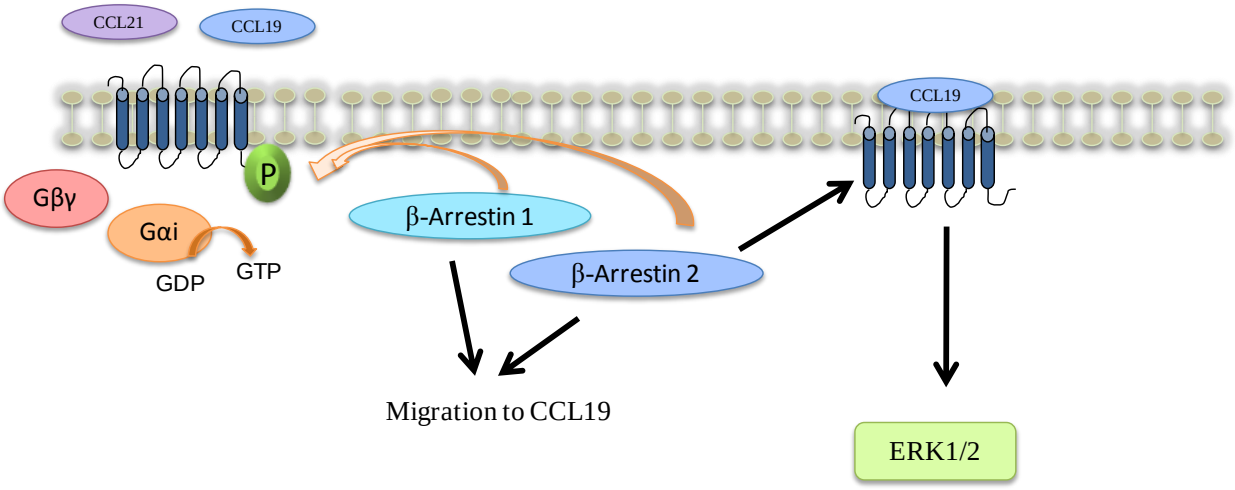

Figure 2. Requirement for arrestins during CCR7 signaling. Both β-arrestin 1 and β-arrestin 2 are required for migration to CCL19, but not CCL21. Depletion of β-arrestin 2 results in a loss of phosphorylation of ERK1/2 in cells stimulated with CCL19.

In addition to MAPKs, GPCRs also are well known for their ability to activate the phospholipase C family of proteins which include PLCA, PLCβ, PLCD and PLCγ. Phospholipase C proteins can regulate cell motility by binding to the actin cytoskeleton as well as interacting with adhesion proteins such as integrins to control migration. In lymphocytes PLCγ1 plays an important role in cytoskeletal rearrangement, cell spreading and migration [56-58]. Activation of PLCγ1 results in hydrolysis of phosphatidyl inositol (4,5) bisphosphate (PIP2) to inositol (1,4,5) triphosphate (IP3) and diaglycerol (DAG) [59]. In T lymphocytes IP3 regulates calcium mobilization and together with DAG can regulate activation of Ras, through the Ras activator RasGRP [60]. Through the activation of Ras, PLCγ1 activation indirectly contributes to activation of MAPK pathways, which are important for controlling cell migration [61]. Another way that PLCγ1 controls migration is that it directly binds to and activates β1 integrins [62]. β1 integrins are important for T lymphocyte transendothelial migration into lymphoid organs. CCR7 phosphorylation of PLCγ1 is required for migration of head and neck cancer cells to CCL19 [63]. However, it is not clear if PLCγ1 activation is important for migration to CCL19 or CCL21 in T lymphocytes.

2.6 Sphingosine-1-Phosphate Receptor 1 and Lymph Node Egress

As naïve T lymphocytes migrate throughout the body traveling into lymphoid organs, they must be able to navigate their way back out and either migrate onto other secondary lymphoid organs or into the periphery to carry out effector functions. The Sphingosine-1-phosphate receptor 1 ($S1P_1$), is expressed on T lymphocytes and is required for lymphocyte egress from the thymus, spleen, Peyer's patches and lymph nodes [64, 65]. The ligand for the

$S1P_1$, sphingosine 1 phosphate (S1P) is expressed at high concentrations in blood (100-300nM) and acts as a gradient to attract cells from lymphoid organs [66]. Interestingly, twenty-four hours following T lymphocyte entry into lymph nodes, T lymphocytes lose the ability to migrate to S1P, correspondingly mRNA for the receptor is decreased 1-10% [64, 65]. Approximately three days following T cell receptor activation, mRNA for $S1P_1$ is increased and cells once again regain the ability to migrate to S1P and rapidly reappear in circulation [65]. During this three day period, T lymphocytes remain in lymph nodes where they interact with dendritic cells that express CCL19, become activated and proliferate (Figure 3). Kruppel Like Factor 2 (KLF2), a transcription factor, regulates $S1P_1$ expression. Similar to $S1P_1$, KLF2 mRNA mirrors the cyclic expression levels of $S1P_1$ during an immune response [67]. However, the precise signal that leads to increased KLF2 transcription resulting in increased $S1P_1$ transcription to allow for lymphocyte egress from lymph nodes remains unclear.

One possible upstream candidate for KLF2 activation is the MAPK, extracellular signal-related kinase 5 (ERK5). ERK5 is expressed in T lymphocytes and is phosphorylated in response to T lymphocyte activation (anti-CD3 and anti-CD28) or IL-7 stimulation [68]. ERK5 directly regulates KLF2 expression in T lymphocytes [68]. Depletion of ERK5 leads to down-regulation of CD62L [68]. CD62L is also known as L-selectin and is an adhesive molecule expressed on the surface of naïve T lymphocytes and plays a role in homing of these cells into secondary lymphoid organs. Consequently, only cells that express high levels of CD62L can efficiently migrate to S1P [65]. Because depletion of ERK5 leads to down-regulation of CD62L on the surface of T lymphocytes, this suggests that migration to S1P could also be diminished. These results suggest a possible role for ERK5 in regulating $S1P_1$ expression. However, anti-CD3, anti-CD28 or IL-7 stimulation of ERK5 does not fully explain $S1P_1$ regulation since ERK5

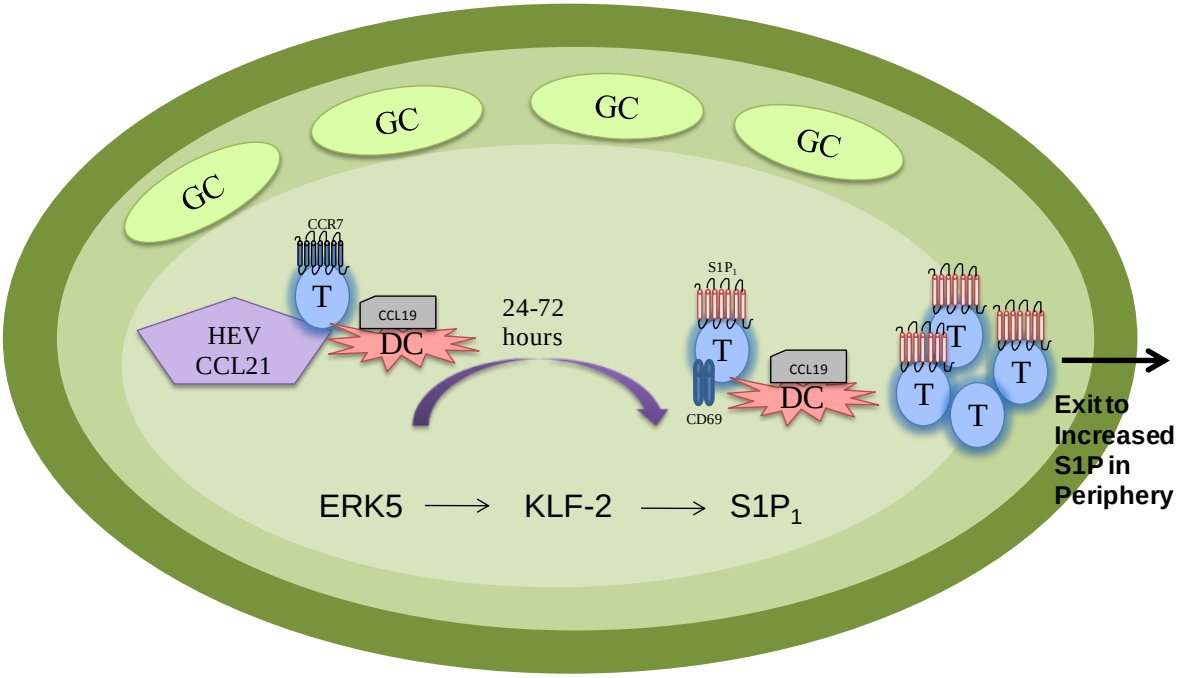

Figure 3. $S1P_1$ regulates T lymphocyte lymph node egress. Naïve T lymphocytes (T) enter into lymph nodes where they begin scanning dendritic cells (DC) that present antigen. Twenty fours hours after entering into the lymph nodes, T lymphocytes down-regulate $S1P_1$ expression. Following brief interactions with the dendritic cells, naïve T lymphocytes will form stable interactions with the dendritic cells that can last 36-48 hours. During this period of time the T lymphocyte will become activated and proliferate. Approximately, 72 hours following T lymphocyte activation $S1P_1$ mRNA is increased and cells regain the ablility to migrate to S1P, which is at a higher concentration in blood. This results in T lymphocytes exiting the lymph node. We proposed that CCL19, which is expressed by dendritic cells provides the signal necessary for increased expression of $S1P_1$. We propose that CCL19 activates ERK5, which increases transcription of KLF-2, which increases transcription of $S1P_1$.

activation is only induced over a short period of time and $S1P_1$ is regulated over the course of days. In contrast, during the ~48 hour time period that T lymphocytes form conjugates with dendritic cells in the lymph nodes, the T lymphocytes are consistently stimulated with CCL19 expressed by dendritic cells. Interestingly, in CCL19 knockout mice retention of both CD4+ and CD8+ cells occurs in lymph nodes during normal homeostasis [34]. In a similar study using a CCL19 antagonist (8-83), cells were also retained in lymph nodes during an allogenic immune response [36]. Furthermore, in CCR7 deficient mice $S1P_1$ levels were also found to be decreased [69] suggesting that CCR7 expression may be important for $S1P_1$ expression. These studies together suggest a possible role for CCR7/CCL19 in lymphocyte egress from lymph nodes (Figure 3).

2.7 Potential Role for CCR7 Signaling in Lymph Metastasis During Breast Cancer Progression

According to the American Cancer Society, breast cancer is the second leading cause of cancer deaths among women in the United States with an estimated 207,090 new cases in women and 1,970 new cases in men occurring in 2010. It is estimated that approximately 40,000 women will lose their lives to breast cancer in the United States this year alone. While the 5 year survival rate has drastically improved to 90%, the rate for survival if the breast cancer has metastasized to distant lymph nodes and organs is a staggering 23% [70].

Metastasis is a complex process whereby a cell detaches from a primary tumor and migrates to a second site. In recent years, it has been recognized that cancers tend to not randomly metastasize to distant sites, but instead favor certain metastatic sites over others. For example, breast cancers typically metastasize to the bone, lung, liver, brain and lymph nodes.

These reoccurring sites of metastasis have been explained due to the expression of chemokine receptors on the surface of breast cancer cells that respond to gradients of chemokines located in target organs. In particular, CXCR4 and CCR7 are expressed on human breast cancer cells and are considered diagnostic biomarkers that predict lymph node metastasis [71-73].

Lymph nodes have not always been thought of as sites of metastasis. Instead lymph nodes have been considered as sentinel sites where metastases simply filter through on their way to other organs. It is not known if tumor cells become trapped in the lymph nodes or continue to metastasize to distant organs. In any case, staging of breast cancer relies heavily on lymph node involvement, with greater involvement correlating to a worse disease outcome [74]. In most breast cancer cases, axillary lymph nodes are removed without a comprehensive understanding if removal is clinically better/worse for patient survival, as lymph nodes are important site for immunological responses [74]. Recent studies found strong correlations that human breast cancers expressing CCR7 specifically metastasize to lymph nodes, which highly express CCR7 ligands, CCL19 and CCL21 [73, 75]. However, a contrasting study determined that CCR7 expression did not correlate with lymph node metastasis in breast cancer [76]. At present, it is unclear if and by what mechanisms CCR7 controls lymph node metastasis in breast cancer.

2.8 CCR7 Signaling in Breast Cancer Cells

As lymphocytes are experts at migrating and entering into tissues, cancer cells take advantage of the same machinery used by lymphocytes to also metastasize and invade tissue. Therefore, we can learn an abundant amount of information about the molecular mechanisms involved in these processes in cancer cells through our knowledge of lymphocyte behavior.

CCR7 is up-regulated in many types of metastatic cancers including gastric, pancreatic, thyroid, endometrial, lung and breast and is implicated in lymph node metastasis [77-81]. Similar to T lymphocytes, CCR7 activates the MAPK, ERK1/2, in cancer cells to induce migration and inhibition of either CCR7 or ERK1/2 results in reduction of migration and invasion [82]. In T lymphocytes PLCγ1 plays an important role in cell migration and β1 integrin activation [56, 57, 62]. In head and neck cancer cells, CCR7 mediated migration requires activation of PLCγ1 [83]. PLCγ1 has also been found to be indispensable for activation of β1 integrins in cancer cells [84]. Additionally, in thyroid carcinoma cells, stimulation of CCR7 by CCL21 increased β1 integrin expression on the cells surface [79].

Similar to T lymphocytes, β1 integrins have been identified as important mediators or breast cancer migration. Compared to the primary tumor, metastatic breast cancer cells display increased expression of β1 integrins on their cells surface [85]. Blocking β1 integrins reverts the malignant phenotype of these breast cancer cells to a normal epithelial phenotype, characterized by well organized actin, organized adherens junctions, deposition of a basement membrane and growth arrest [85]. Additionally, use of AIIB2 a β1 integrin functioning antibody, in combination with radiation therapy significantly enhanced the effects of the radiation therapy and reduced the tumor size in human cells and *in vivo* [86]. A better understanding of how CCR7 regulates β1 integrin up-regulation or activation can advance our understanding of how breast cancer cells become metastatic and invade surrounding tissue. Finally, a better understanding of the role of CCR7 in mediating breast cancer metastasis to lymph nodes is necessary in order to design novel therapeutic strategies targeting this pathway.

CHAPTER 3- CCR7/CCL21 MIGRATION ON FIBRONECTIN IS MEDIATED BY PLCγ1 AND ERK1/2 IN PRIMARY T LYMPHOCYTES

3.1 Abstract

C-C Chemokine receptor 7 (CCR7) binds to its cognate ligand, CCL21, to mediate the migration of circulating naive T lymphocytes to the lymph nodes. T lymphocytes can bind to fibronectin, a constituent of lymph nodes, via their β1 integrins, which is a primary mechanism of T lymphocyte migration; however, the signaling pathways involved are unclear. We report that Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) is required for T cell migration on fibronectin in response to CCL21 with a rapid (within 2 minutes) and transient phosphorylation of ERK1/2. Conversely, prevention of ERK1/2 phosphorylation by inhibition of its kinase, MEK, prevented T lymphocyte migration. Previous studies have suggested that Phospholipase C gamma 1 (PLCγ1) can mediate phosphorylation of ERK 1/2, which is required for β1 integrin activation. Paradoxically, we found that inhibition of PLCγ1 phosphorylation by the general PLC inhibitor, U73122, was associated with an enhanced phosphorylation of ERK1/2 and reduced migration of T lymphocytes on fibronectin. To further characterize the relationship between ERK1/2 and PLCγ1, we reduced PLCγ1 levels by 85% using shRNA and observed a sustained phosphorylation of ERK1/2 and a significant reduction in CCR7 mediated migration of T lymphocytes on fibronectin. In addition, we found that inhibition of ERK 1/2 phosphorylation by U0126 resulted in a decreased phosphorylation of PLCγ1 suggesting a feedback loop between ERK 1/2 and PLCγ1. Overall, these results suggest that the CCR7 signaling pathway leading to T

lymphocyte migration on fibronectin is a β1 integrin dependent pathway involving transient ERK1/2 phosphorylation, which is modulated by PLCγ1.

3.2 Introduction

G-protein coupled receptors (GPCRs) are responsive to many types of stimuli such as hormones, neurotransmitters and chemoattractants. Ligand activation of a GPCR causes a conformational change in the receptor that leads to an exchange of GDP for GTP in the Gα subunit and dissociation of Gα from the Gβγ subunits. The subunits then initiate downstream signaling events to regulate activation of adenylyl cyclase or phospholipase C (PLC) [42]. C-C Chemokine Receptor 7 (CCR7), a G-protein coupled receptor, is expressed on naive T lymphocytes and is required for migration into and within lymph nodes. CCR7 binds to two ligands CCL19 and CCL21. CCL21 is expressed in high endothelial venules, the entry route into lymph nodes [28]. However, it is currently unknown how T lymphocytes signal through CCR7 to mediate migration via β1 integrins in response to CCL21.

Integrins, which are key mediators of lymphocyte adhesion and migration, are heterodimeric adhesion proteins, consisting of an α and the β subunit, for which they are named. β1 integrins which are expressed by T lymphocytes and bind to VCAM, collagen and fibronectin [87]. Fibronectin is a major component of the lymph node and is highly expressed in the cortex in the presence of fibroblastic recticular cells, which express CCL21[17, 88]. This network provides an environment that allows CCR7 expressing T lymphocytes to migrate throughout the lymph node.

Phospholipase C (PLC) is a downstream target of Gβγ signaling and is important for T cell migration [89]. PLCγ binds to and activates β1 integrins [62]. Furthermore, PLCγ1 is required

for adhesion to fibronectin and important for β1 integrin mediated cell migration [63, 84]. PLCγ1 was recently shown to be phosphorylated by CCR7 in head and neck cancer cells and was necessary for migration of squamous cell carcinoma of head and neck cells [83].

GPCRs can activate the downstream effector mitogen activated protein kinases (MAPK) to regulate chemotactic migration [53, 90]. In HEK293, CCR7 transient transfectants, stimulated with CCL19 rapidly activated extracellular related kinase 1/2 (ERK1/2), while CCL21 had a minimal effect on ERK1/2 phosphorylation [46]. In primary B lymphocytes that express endogenous CCR7, CCL21 mediated activation of ERK1/2 [53]. These contrasting studies illustrate the importance of determining the role of ERK1/2 in the migration of primary T lymphocytes to CCL21.

Therefore, we examined the molecular mechanisms that are required for β1 integrin activation and β1-mediated migration. In primary T lymphocytes we have found that CCL21 promotes activation of PLCγ1 and transient activation of ERK1/2, which are required for β1 integrin activation in response to CCL21. Loss of activation of either PLCγ1 or ERK1/2 prevented migration via β1 integrins to CCL21. These results indicate that CCL21 activation of CCR7 promotes activation of β1 integrins leading to migration following phosphorylation of PLCγ1 and ERK1/2.

3.3 Results

3.3.1 Primary Lymphocytes Differentially Migrate to CCL21 on Fibronectin

Naïve T lymphocytes which express CCR7, enter into lymph nodes in response to CCL21 expressed in high endothelial venules. To define the molecular mechanisms that control CCL21

mediated T lymphocyte migration we used chemotaxis assays. Naïve T lymphocytes were induced to migrate over a physiological range of CCL21 concentrations (0-400nM). We found that naïve T lymphocytes migrated to CCL21 at 2μM concentration on polycarbonate membranes (Figure 4A). To understand how T lymphocytes migrate via β1 integrins we pre-incubated the polycarbonate membranes with the β1 integrin ligand, fibronectin. Lymphocytes were induced to migrate over a wide physiological concentration to 0-400nM CCL21, with the greatest migration occurring at 40nM (Figure 4B). These results implicate β1 integrins in T lymphocyte migration to CCL21.

3.3.2 ERK1/2 is required for Migration to CCL21

GPCRs can activate extracellular signal related kinase (ERK1/2) in order to mediate migration of human primary macrophages and human osteosarcoma cells [90, 91]. To examine the role of ERK1/2 in the migration of naïve T lymphocytes to CCL21 via their β1 integrins, we examined migration to CCL21 in the presence of the MEK inhibitor UO126. Under these conditions naïve T lymphocytes failed to migrate on fibronectin compared with controls treated with vehicle (DMSO) (Figure 5A). To confirm that UO126 blocked MEK activation of ERK1/2, we used western blots. As expected, UO126 blocked ERK1/2 phosphorylation in cells treated with CCL21 (Figure 5B). PLCγ can mediate phosphorylation of ERK1/2 [92, 93]. To examine the contribution of ERK1/2 to activation of PLCγ1, we probed for PLCγ1 phosphorylation. Unexpectedly, we found that phosphorylation of PLCγ1 was decreased (Figure 5B). From these results we concluded that rapid ERK1/2 activation is required for migration to CCL21.

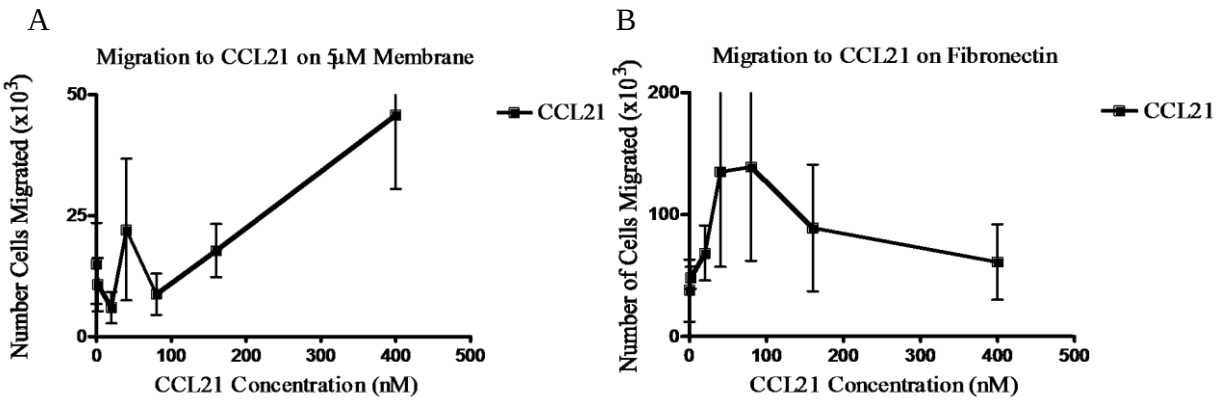

Figure 4. Migration of primary cells to CCL21 on fibronectin. A) Migration of primary naïve T lymphocytes to 0-400nM CCL21 on 5um membranes incubated in sfRPMI (n=3), $p < 0.01$. B) Migration of primary naïve T lymphocytes to 0-400nM CCL21 on 5um membranes incubated in 10μg/ml fibronectin (n=4), $p < 0.004$.

Furthermore, PLCγ1 phosphorylation may be mediated by activated ERK1/2 to regulate CCL21 mediated migration.

Cell migration requires regulated integrin adhesion, which is mediated by activation, followed by integrin de-adhesion [94, 95]. Therefore, we questioned the role of ERK1/2 in β1 integrin activation. To this end we pretreated lymphocytes with UO126 or vehicle (DMSO) and measured levels of activated β1 integrins following activation with CCL21. Lymphocytes were stained with an activation specific antibody, 12G10 [96]. Levels of activated β1 integrins were analyzed by flow cytometry. We found that lymphocytes treated with UO126 displayed similar β1 integrin activation compared to controls (Figure 5C). From this data we concluded that β1 integrin activation in T lymphocytes in response to stimulation of CCR7 with CCL21 is not regulated by ERK1/2 phosphorylation.

3.3.3 PLC is required for Migration on Fibronectin to CCL21

As mentioned, PLCγ1 binds to and activates β1 integrins [62]. Since loss of ERK1/2 phosphorylation correlated with decreased PLCγ1 phosphorylation and reduced migration to CCL21, we questioned if PLC was necessary to control migration to CCL21. To determine if PLC was necessary for migration, we used chemotaxis assays in the presence of U73122, a universal PLC inhibitor, or the U73343 control. To confirm that U73122 blocked activation of PLCγ in T lymphocytes in response to CCL21 stimulation, we assayed for phosphorylation of PLCγ1 by western blot. Lymphocytes pretreated with U73122 displayed decreased phosphorylation of PLCγ1. In contrast, phosphorylation of ERK1/2 was increased compared to controls (Figure 6B). When T lymphocytes were pre-incubated with U73122, migration to

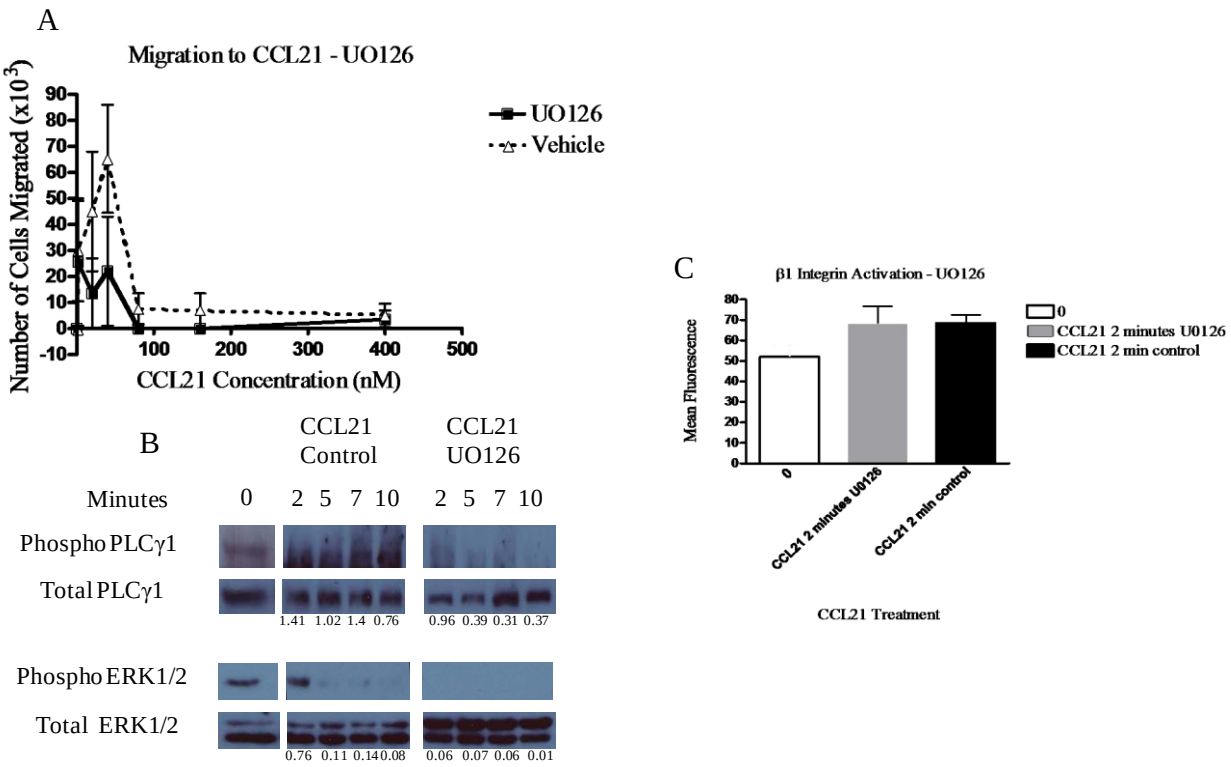

Figure 5. Inhibition of ERK1/2 decreases migration of T lymphocytes to CCL21. Primary naïve T lymphocytes were treated with 1uM UO126 or DMSO (control) for 90 minutes and were migrated to 40nM CCL21across a membrane pre-incubated in 10µg/ml fibronectin. A) Cells pretreated with UO126 migrated to CCL21 (n=3), $p < 0.03$. B) Cells stimulated with CCL21 probed for ERK1/2 and PLCγ1 phosphorylation and total (n=3). Densitometric analysis of PLCγ1 and ERK1/2 was performed using ImageJ software. C) Cells were treated with UO126, stimulated with 40nM CCL21 and assayed for activated β1 integrins on the cell surface (n=3), $p < 0.41$.

CCL21 was reduced (Figure 6A). From these results we concluded that activation of PLC mediates migration of T lymphocytes to CCL21.

3.3.4 PLCγ1 is Necessary for β1 Integrin Migration and Activation

Because U73122 is a universal inhibitor of PLC, to determine the role PLCγ1 in the migration of naïve T lymphocytes via β1 integrins, we used PLCγ1 shRNA to specifically target and knock down levels of PLCγ1 in primary human T lymphocytes. Lymphocytes were transiently transfected with PLCγ1 shRNA or control shRNA and assayed for migration and β1 integrin activation. We found that total PLCγ1 levels were reduced approximately 85% in the shRNA transfected lymphocytes (Figure 7A). T lymphocytes that were depleted of PLCγ1 failed to migrate on fibronectin compared to controls (Figure 7B). In addition, loss of PLCγ1 led to a failure to activate β1 integrins in response to stimulation with CCL21 as determined by flow cytometry (Figure 7C). Since in response to U73122, we observed an increase in ERK1/2 phosphorylation, we questioned if this was due to inhibition of PLCγ1. Similarly, we observed that knock down of PLCγ1 resulted in a trend towards increased ERK1/2 phosphorylation compared to controls (Figure 7D and 7E). From these results we concluded that PLCγ1 is required for transient ERK1/2 phosphorylation and activation of β1 integrins resulting in migration of naïve T lymphocytes to CCL21 on fibronectin.

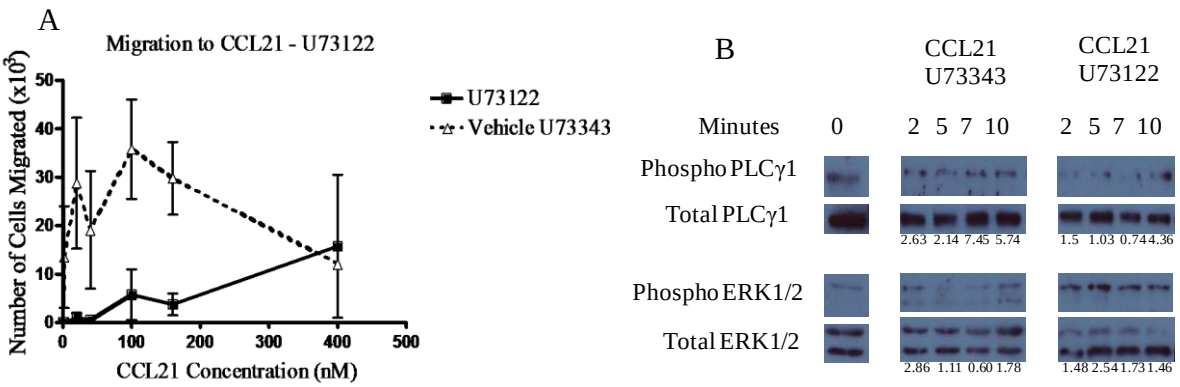

Figure 6. Migration to CCL21, PLCγ1 and ERK1/2 phosphorylation is decreased upon U73122 treatment. Primary naïve T lymphocytes were pretreated with 2.0uM U73122 for 20 minutes and migrated to 40nM CCL21 on membranes preincubated with 10μg/ml fibronectin. A) Cells treated with U73122 were migrated on fibronectin to CCL21. (n=3), $p < 0.03$. B) Cells stimulated with CCL21 were probed for PLCγ1 and ERK1/2 total and phosphorylation (n=3). Densitometric analysis of PLCγ1 and ERK1/2 was performed using ImageJ software.

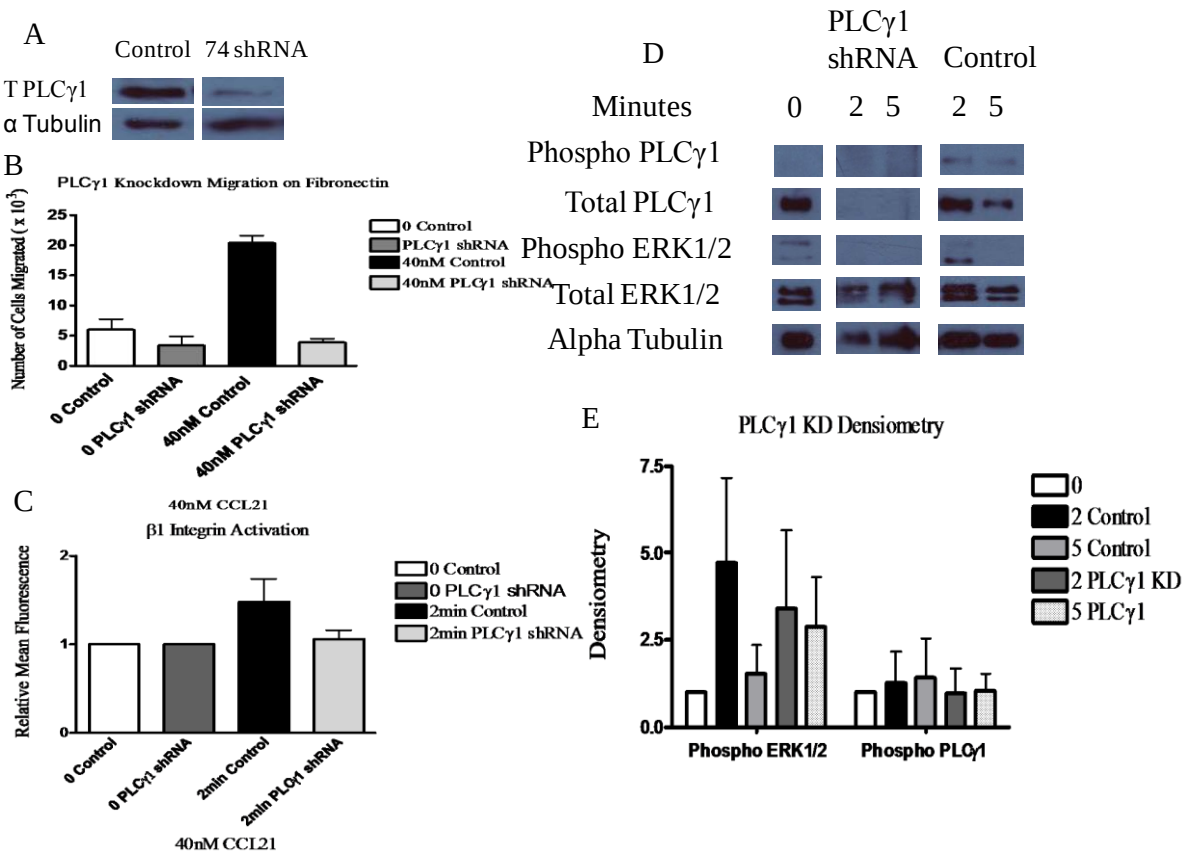

Figure 7. PLCγ1 shRNA reduces migration to CCL21, β1 integrin activation and increases ERK1/2 phosphorylation. Primary naïve T lymphocytes were transiently transfected (LipoD 293) with 8ug/ml PLCγ1 shRNA or control shRNA and incubated at 37°C, 5%CO_2 for 72 hours before use. A) Human primary T lymphocytes were depleted of PLCγ1 approximately 85% . B) PLCγ1 knock down cells were migrated on fibronectin to 40nM CCL21 (n=3). $p < 0.002$. C) β1 integrin activation (n=3), $p < 0.03$. D) Phosphorylation of ERK1/2 and PLCγ1 in response to CCL21 stimulation. (n=3). E) Graphical Representation of western. Densitometric analysis of PLCγ1 and ERK1/2 was performed using ImageJ software.

3.3.5 Gαi Mediates Migration to CCL21

To examine the signaling events upstream of PLCγ1 we investigated the role of G proteins in the activation of PLCγ1. CCR7 couples to Gαi to initiate downstream signaling events and is required for migration to CCL21 [28]. Therefore, to examine signaling events more proximal to the membrane that mediates activation of PLCγ1, we pretreated lymphocytes with pertussis toxin. As expected, lymphocytes pre-treated with pertussis toxin failed to migrate to CCL21 compared to controls (Figure 8A). Therefore, to determine the role of Gαi in signaling through CCR7 to mediate activation of PLCγ1 and ERK1/2 in the presence of pertussis toxin, we assayed for changes in phosphorylation of PLCγ1 and ERK1/2. In pertussis toxin treated lymphocytes phosphorylation of ERK1/2 and PLCγ1 was lost (Figure 8B). These results indicate that CCR7 coupling to Gαi is necessary for activation of PLCγ1 and ERK1/2 mediated migration on fibronectin. Furthermore, activation of Gαi is required to induce phosphorylation of both PLCγ1 and ERK1/2.

To determine the extent to which Gαi affects β1 integrin activation in naïve T lymphocytes, we pretreated lymphocytes with pertussis toxin, stimulated with 40nM CCL21 and assayed by flow cytometry for activated integrins on the cell surface. We observed that loss of Gαi activation had no effect on β1 integrin activation, although cell migration to CCL21 was lost when compared to controls (Figure 8C). Since cell migration requires control of integrin adhesion (activation) and de-adhesion (de-activation), from these studies we concluded that signaling through Gαi leads to cycling of adhesion and de-adhesion states. These results suggest that Gαi is important for migration to CCL21, but not for the initial activation of β1 integrins in response to CCL21 stimulation.

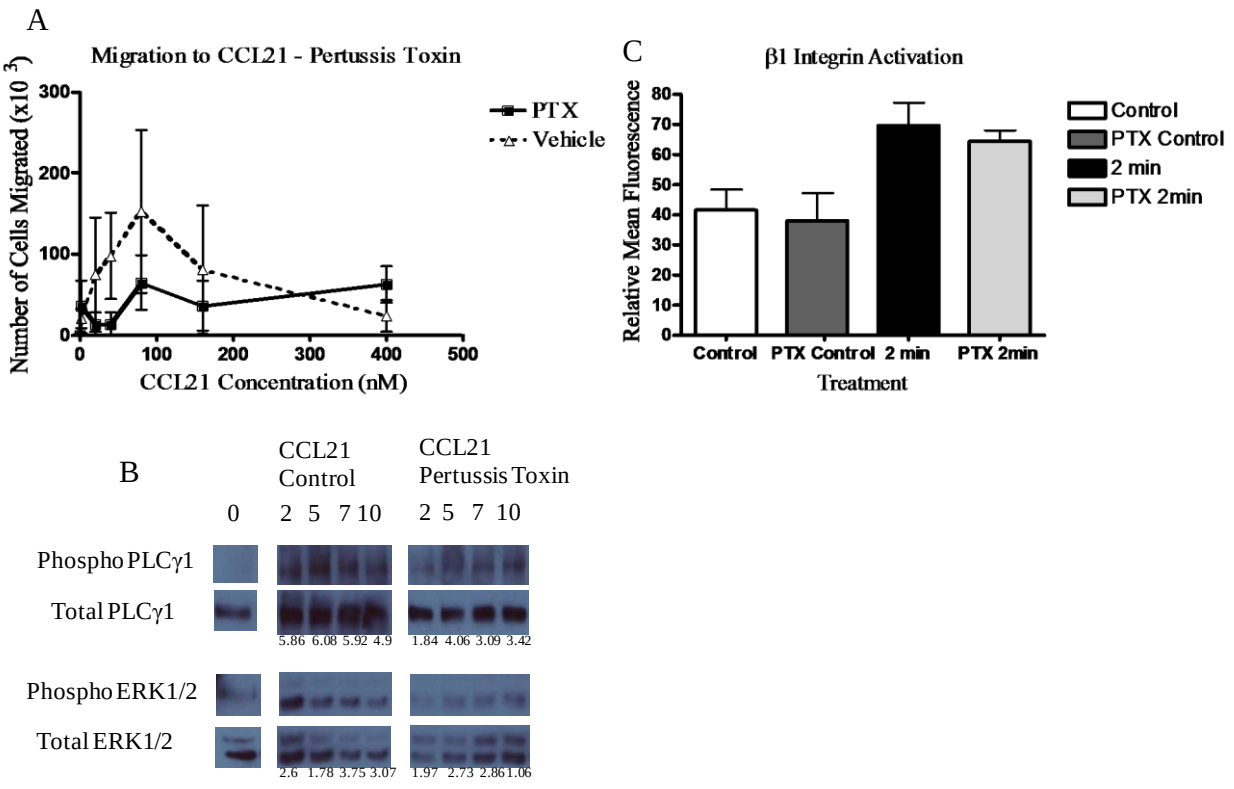

Figure 8. Inhibition of Gαi decreases cell migration to CCL21, phosphorylation of ERK1/2 and PLCγ1 and β1 integrin activation. Cells were pretreated with 100ng/ml pertussis toxin for 2 hours and were migrated across a membrane incubated in 10μg/ml fibronectin to 40nM CCL21. A) Cells were pretreated with pertussis toxin and migrated to 40nM CCL21 (n=3), $p < 0.04$. B) Cells pretreated with pertussis toxin probed for total and phosphorylation of PLCγ1 and ERK1/2 (n=3). Graphical averages of three independent experiments. Densitometric analysis of PLCγ1 and ERK1/2 was performed using Image J software. C) Cells were stimulated with 40nM CCL21, incubated with 3ug/ml 12G10 anti-human β1 antibody, labeled with anti-mouse FITC secondary and assayed by flow cytometry. (n=3) $p < 0.122$.

3.4 Discussion

CCR7 is expressed on B lymphocytes, dendritic cells, natural killer cells, T regulatory cells and naïve T lymphocytes and is required for their migration into and within lymph nodes. CCR7 binds to two ligands, CCL19 and CCL21. Although many studies have described CCL19 signaling through CCR7, little is known about how CCL21 signals through CCR7 leading to migration [97-100]. Here we show that CCL21 stimulation of CCR7 leads to migration on fibronectin, which requires activation of Gαi and phosphorylation of PLCγ1 and ERK1/2.

High endothelial venules as well as lymph nodes express substantial amounts of fibronectin (1). As lymphocytes enter into and migrate through lymph nodes they use β1 integrins for adhesion and migration (2). Because CCL21 is required for lymph node entry as well as migration within the lymph nodes, we wanted to define the CCL21 mediated mechanisms that control β1 integrin-mediated migration on fibronectin. In this study we provide evidence that CCL21 induces β1 integrin activation and promotes migration on fibronectin via PLCγ1 and ERK1/2 activation.

GPCRs are well known for their ability to activate MAPKs [52, 101]. MAPKs are involved in many cellular processes, including migration [102-104]. Several groups have reported that stimulation of CCR7 by CCL21 phosphorylates the MAPK ERK1/2 in primary murine T lymphocytes, B lymphocytes and HEK293 CCR7 transfected cells [46, 53, 54]. However, from these studies it is difficult to interpret what if any role ERK1/2 plays in migration of T lymphocytes. In this study similar to previous studies, we report that ERK1/2 is rapidly and transiently phosphorylated in response to CCR7 activation by CCL21. In addition, we find that ERK1/2 is required for migration to CCL21 via CCR7 on the β1 integrin ligand, fibronectin. β1

integrin activation can result in ERK1/2 phosphorylation as a result of "outside in" signaling [105], however we also find that transient ERK1/2 phosphorylation can mediate migration to CCL21 via CCR7 by signaling to β1 integrins by "inside out" signaling.

Transient activation of ERK1/2 has been shown to be important for aortic smooth cell migration [55]. We used the MEK inhibitor UO126, to demonstrate that phosphorylation of ERK1/2 is important for T cell migration in response to CCL21. In addition, using PLCγ1 shRNA, we found that ERK1/2 phosphorylation was sustained which correlated with decreased migration to CCL21. Taken together, these results suggest rapid phosphorylation followed by a rapid decrease in phosphorylation of ERK1/2 is important for migration to CCL21 in T lymphocytes.

Stimulation of human epidermoid carcinoma cells with 12 (S) HETE results in PLCγ1 phosphorylation and downstream activation of ERK1/2 via pertussis toxin sensitive signaling events [106]. In our study we found that transient phosphorylation of ERK1/2 is lost following reduction of PLCγ1 levels, suggesting that PLCγ1 contributes to the transient phosphorylation of ERK1/2. Taken together we determined that not only is ERK1/2 important for migration, but PLCγ1s also important for regulating states of ERK1/2 activation. These states of ERK1/2 activation may also play an important role in β1 integrin de-activation.

As mentioned, cell migration requires regulated integrin adhesion (activation) followed by de-adhesion (in-activation). Interestingly, we found that Gαi activation was not necessary for β1 integrin activation however, pertussis toxin inhibited migration to CCL21. Similarly, we found that UO126 also had no effect on β1 integrin activation, but also inhibited migration to CCL21. Because pertussis toxin inhibited phosphorylation of ERK1/2, we speculated that Gαi and ERK1/2 are not required for the initial activation of β1 integrins, however they are required for

de-adhesion (in-activation) of β1 integrins, which leads to the loss of migration as seen with pertussis toxin and UO126. Depleted PLCγ1 resulted in increased and sustained ERK1/2 phosphorylation suggesting that it is the de-phosphorylation of ERK1/2 that is important for β1 integrin recycling and migration to CCL21.

T lymphocytes are crucial mediators of inflammation, autoimmune disorders, allergic disease and cancer. Migration is not only important for a normal functioning immune system, but also important in disease. Therefore, it is important to understand the molecular mechanisms of how T lymphocytes migrate into and throughout lymph nodes to become effector cells to carry out immune responses in the periphery. This study is an important step in further understanding the molecular mechanisms of how T lymphocytes migrate to CCL21 and therefore could provide identification of inhibitors that could specifically target T cell entry and migration within lymph nodes in order to regulate the immune response.

3.5 Experimental Procedures

3.5.1 Mice, Primary Human Lymphocytes and Reagents

C57BL/6 mice were purchased from Jackson Labs. Primary human T lymphocytes were isolated from volunteer donors under an approved protocol, in accordance with policies and procedures of the human subjects protection program at the University of Kansas Medical Center. UO126 (Calbiochem), U73122 and U73343 (Biomol) and pertussis toxin (List Biological Laboratories, Inc), human PLCγ1 shRNA (Origene) and species specific CCL19 and CCL21 (R&D) were purchased. 12G10 anti human activated beta 1 integrin antibody was generously provided by Dr. Martin Humphries at The University of Manchester.

3.5.2 T Lymphocyte Isolation

Murine splenocytes were harvested and purified by negative selection (EasySep). Human blood was collected from volunteer donors and peripheral blood mononuclear cells were isolated by Ficoll-paque (GE Healthcare Life Sciences) gradient. Whole blood was transferred to 50ml conical tubes and diluted at a 1:1 ratio with PBS+Ca^{2+}/Mg^{2+} (Cellgro). In a separate 50ml conical tube 15ml of ficoll was layered with 35ml of whole blood/PBS+Ca^{2+}/Mg^{2+}, and centrifuged for 20 minutes at 2,000rpm without the brake. The middle layer containing lymphocytes was removed, washed and pelleted. The cell pellet was resuspended in 2ml of ACK lysis buffer (Lonza), incubated for 5 minutes at 37°C then rinsed with PBS. T lymphocytes were negatively selected by EasySep kit (Stemcell Technologies) according to manufacturer's protocol. Lymphocytes isolated from C57BL6 mice or human donors were maintained in RPMI-1640 (Invitrogen), 10% heat inactivated fetal bovine serum (Hyclone), 2nM L-glutamine (Invitrogen) in a humidified atmosphere at 37°C and 5% CO_2 for use within 3 days of isolation.

3.5.3 Chemotaxis Assays

All chemotaxis assays were carried out using a 48 well chemotaxis chamber (Neuroprobe). Lower wells were loaded with 0-400nm of CCL21 in serum free RPMI-1640 (sfRPMI) and were separated from the upper wells, which contained lymphocytes, by a 5 µm pore-nitrocellulose (Neuoprobe) membrane that had been pre-incubated in either sfRPMI or 10µg/ml fibronectin (Sigma). C57BL6 splenocytes or primary human T lymphocytes ($5x10^5$) were migrated across the nitrocellulose membrane in a humidified, 37°C, 5% CO_2 chamber for 2

hours to gradients of CCL21. Chambers were disassembled, lymphocytes in the bottom chamber were collected, fixed in 2% paraformaldehyde and counted by hemacytometer. Assays were performed in duplicate and replicated 3 times.

3.5.4 Western Blots

Lymphocytes were pre-treated in serum free media with either UO126 (1μM) for 90 minutes, U73122 (2μM) for 20 minutes or pertussis toxin (100ng/ml) for 2 hours at 37°C. Lymphocytes were then stimulated with 10nM CCL19 or CCL21 for 0,2,5,7, and10 minutes. Lymphocytes were lysed in radio immunoprecipitation assay buffer (RIPA) (100mM Tris pH 8.0, 150mM NaCl, 2% Nonidet P40, 1% sodium deoxycholate, 0.2% SDS) supplemented with protease inhibitor cocktail (Sigma), 1mM sodium ortho-vanadate, 1mM sodium pyrophosphate, 10mM sodium fluoride (Sigma) and 1mM β-glycerophosphate (Sigma) for 15 minutes on ice. Lysates were sheared and insoluble material removed by centrifugation @ 14,000 x g for 20 minutes. Supernatants ($5x10^5$ cell equivalents per timepoint) were mixed with Laemmli sample buffer, fractionated on 7.5% SDS poly acrylamide gels and transferred to PVDF membranes. Membranes were pre-incubated in 5% milk/Tris buffered saline (50mM Tris-HCl pH→7.5, 150mM NaCl) 0.1% Tween-20 (TBST) or 5% BSA/TBST (Sigma) and probed with anti-phospho ERK1/2, anti-total ERK1/2, anti-total PLCγ1 or anti-phospho PLCγ1 (Cell Signaling Technologies). Primary antibodies were detected with HRP-conjugated anti species specific IgG (Pierce) and immune complexes were visualized with chemilluminence Supersignal West Femto (Pierce). Membranes were stripped using Blotfresh (Blotfresh (Signagen)), rinsed in 1X TBST, blocked and re-probed. Protein bands were quantified using ImageJ software.

3.5.5 PLCγ1shRNA Transfection

Primary human T lymphocytes were transiently transfected with PLCγ shRNA or control shRNA using LipoD293 according to manufacturers' instructions (Signagen). Briefly, in separate tubes PLCγ1 and control (8μg) shRNA were diluted into DMEM. In a second set of tubes LipoD293 was diluted into DMEM. Diluted LipoD293 was added to the diluted shRNA, incubated for 15 minutes and the LipoD293 complexes were added to the lymphocytes. Lymphocytes were incubated with the complexes in complete media for 72 hours at 37°, 5%CO2 before use in assays.

3.5.6 Beta 1 Integrin Activation Assay

Lymphocytes were treated with UO126, pertussis toxin or transfected with PLCγ1 shRNA as described previously. Lymphocytes were unstimulated (0 minutes) or stimulated (2 minutes) with 10nM CCL19 or CCL21. Following stimulation lymphocytes were submerged into 1ml of ice cold 1X PBS. Lymphocytes were labeled with 10μg/ml 12G10 on ice for 30 minutes, washed in 1X PBS two times, incubated with fluorescein isothiocyanate (FITC) conjugated anti-mouse secondary antibody (Jackson Laboratories) for 30 minutes and washed in 1X PBS two times. Lymphocytes were fixed in 2% paraformaldehyde and analyzed by the FACS Calibur.

3.5.7 Statistics

All migration and β1 integrin activation assays are shown as two tailed, unpaired student's *t* test. A p value of <0.05 was considered statistically significant.

CHAPTER 4 - CCR7/CCL19 MEDIATES T LYMPHOCYTE EXPRESSION OF EDG-1

4.1 Abstract

T cells continually cycle between the blood, tissues and lymph to carry out immune surveillance and to mediate inflammatory responses. While CCR7 controls lymphocyte recruitment to lymph nodes, its role in the egress of cells from the lymph nodes remains unexplored. Here, we report a critical role for CCR7 and its ligand CCL19 in activating extracellular regulated kinase 5 (ERK5) and up-regulating the endothelial differentiation gene 1 (EDG1) transcription factor Kruppel-Like factor 2 (KLF2) in naïve T cells. Further, we demonstrate that exit of thymocytes from the thymus induces expression of EDG1, since this migration is inhibited in the presence of FTY720. Using shRNA to knock down expression of ERK5 we demonstrate that signaling from CCR7 is required to induce EDG1 expression, and therefore exit of T cells from the lymph nodes. Thus, we define a novel signaling pathway which regulates T cell migration via CCR7/CCL19.

4.2 Introduction

Following maturation, naïve T cells exit the thymus, travel through the circulation and enter lymph nodes via high endothelial venules (HEV) [107]. Well-defined immunological events that control T cell trafficking to and through lymph nodes are regulated by selectins, integrins, and chemokine receptors [108, 109]. Lymph node entry is mediated primarily via chemotaxis of C-C Chemokine receptor 7 (CCR7) expressing naïve T cells to CCL21 [31, 35], a

chemokine which is expressed along the HEV. In addition to CCL21, cells expressing CCR7 respond to CCL19. Stromal cells within the T zone express both CCL19 and CCL21, while CCL19 is also expressed on mature dendritic cells [16, 110, 111]. Although both ligands are found in different regions of lymph nodes, physiologically distinct roles for each of the ligands remain mostly undefined. Recent studies by our laboratory and others, however, have revealed that T cells respond differentially to CCL19 and CCL21 [112-115].

Studies to define roles for CCL19 and CCL21 have been carried out in paucity of lymph node T cell (*plt)* mice, a spontaneous mutant that lacks functional CCL19 and CCL21 [31, 116, 117]. The absence of both ligands makes it difficult to discriminate specific, individual functions for CCL19 or CCL21. Therefore, as expected, the phenotype of the *plt* mouse is similar to that of the $CCR7^{-/-}$ mouse [29]. Further efforts to define individual roles for CCR7 ligands have used ectopically expressed CCL19 or CCL21 and have revealed that CCL21 promotes lympho-neogenesis more efficiently than CCL19 [35, 113]. More recently the $CCL19^{-/-}$ mouse, in which the CCL19 locus has been homozygously deleted, appeared normal, although there was a statistically significant increase in the number of lymphocytes in the lymph nodes of the $CCL19^{-/-}$ mouse when compared to a congenic wild type strain [34]. Similarly, adoptively transferred cells were trapped in the lymph nodes of mice treated with the CCL19 specific antagonist ELC_{8-83} [36]. Normally, T cells exit the lymph nodes via the endothelial differentiation gene 1 (EDG-1), a receptor for sphingosine-1 phosphate [65]. Because in the absence of CCL19 stimulation T cells fail to exit the lymph nodes, it was unclear whether the CCR7 receptor could be involved in regulating egress of cells from the lymph nodes via EDG-1. In contrast, the role of CCL21 appears to be lymph node recruitment, since in the presence of a CCL21-specific antagonist, T cells failed to migrate to secondary lymphoid organs [37].

In T cells, transcription of EDG-1 is regulated by the Kruppel like factor 2 (KLF-2) [118] while expression of KLF-2 is thought to be regulated by extracellular regulated kinase 5 (ERK5) [68]. ERK5 (Big-Mitogen Activated Protein Kinase 1 (BMK1)) belongs to the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases. ERK5 contains a C-terminal trans-activation domain that allows it to directly regulate [119, 120] the myocyte enhancing factor 2 (MEF2) family of transcription factors [121]. Via MEF2 transcription factors, ERK5 promotes transcription of KFL2[68] and KLF2 is required for the expression of EDG1 [118]. How this signaling pathway is regulated in T cells once they enter the lymph node is unknown.

In this manuscript, to characterize the signaling events that control EDG-1 expression we used the human HuT78 T cell line to examine the contribution of CCL19 to the regulation of the expression of EDG-1. Examination of this pathway revealed that CCL19 mediates activation of the extracellular regulated kinase 5 (ERK5), which leads to increased levels of expression of Kruppel-like factor 2 (KLF2). Following prolonged stimulation with CCL19 we observed increased migration of cells to EDG-1. Knockdown of ERK5 expression by shRNA inhibits KLF2 expression, and results in a loss of migration to S1P. We used murine primary T cells and targeted siRNA to confirm this pathway *in vivo*. To determine if CCL19 expression by activated dendritic cells, mediates recruitment of naïve T cells and the resultant expression of EDG1, we blocked signaling via CCL19 by using CCL19 shRNA on activated dendritic cells *in vitro* and a CCL19 antagonist, ELC_{8-83} in an adoptive transfer assay, *in vivo* [36]. We found that in the absence of CCL19, ovalbumin-primed dendritic cells failed to form conjugates with ovalbumin specific T cells *in vitro. In vivo*, in the presence of the CCL19 antagonist, naïve T cells failed to

up-regulate EDG-1 and therefore did not leave the lymph nodes. Taken together, these studies define a novel pathway in which CCL19 activates up-regulation of EDG-1.

4.3 Results

4.3.1 CCL19 Stimulation of Thymocytes Leads to EDG1 Mediated Migration

In fetal thymic organ cultures, migration of thymocytes from the thymus requires a minimum of eight hours stimulation with CCL19. The thymocytes do not migrate to CCL21. In contrast, thymocytes dissociated from these fetal thymic organ cultures migrate robustly to both CCL19 and CCL21 during a 90 minute chemotaxis assay in transwells, *in vitro* [122]. Since the migration from the intact fetal thymic organ cultures in response to stimulation with CCL19 is delayed for eight hours, we hypothesized that this migration is promoted by transcriptional up-regulation of a second protein(s). EDG-1 is a candidate protein, since it is required for migration of thymocytes from the thymus [65] and the ligand for EDG-1, sphingosine-1 phosphate, is at a concentration of about 100nM in [123] fetal thymic organ culture media. This concentration is sufficient to induce EDG-1 migration of primary T cells [65]. To examine the role of EDG-1 in CCL19 induced migration of T cells, we used fetal thymic organ cultures, treated with the EDG-1 receptor antagonist FTY720 [65, 124] (Figure 9A). Antagonizing the EDG1 receptor with the FTY720 blocked T cell migration in response to stimulation with CCL19, and the migration of these thymocytes to CCL19 was reduced to the level of the cells treated without ligand (Figure 9B). These studies demonstrate that treatment of fetal thymic organ cultures with CCL19, leads to the migration via a receptor that can be antagonized by FTY720.

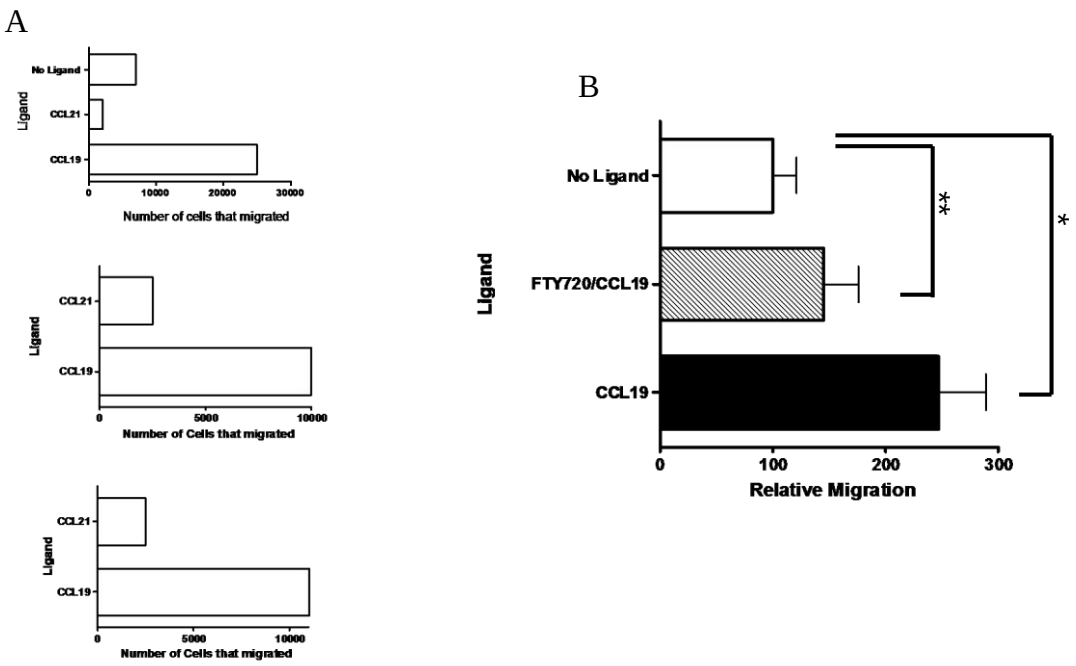

Figure 9.Thymic migration of T cells to CCL19 can be blocked by FTY720. A.) Representative migration assay of day 15.5 thymus. B) Inhibition of migration of thymocytes from a day 15. 5 thymus in the presence of FTY720. *p=0.0018; **p=0.675 students T test.

4.3.2 Activation of CCR7 via CCL19 but not CCL21 Leads to Phosphorylation of ERK5

Our attempts to block the migration of fetal thymocytes via retroviral transduction of the fetal thymic organ cultures were thwarted, since transduction with the control virus, led to an increase in ERK5 and a concomitant increase in the number of cells that migrated to CCL19 (data not shown). Therefore, to define the CCR7/CCL19-regulated signaling events in T cells, we used the CCR7 expressing human T cell line, HuT78[112]. To determine if EDG1 was up-regulated in the HuT78 human T cell line following stimulation with CCL19, we examined HuT78 migration to 10nM sphingosine-1-phosphate following stimulation with 100nM CCL19 or CCL21 for 24, 48, 72 or 96 hours. Only in response to CCL19 did we observe migration at 48 and 72 hours (Figure 10A). In the presence of CCL21 cells migrated to the same extent as the controls. Semi-quantitative RT-PCR was used to measure levels of EDG1 expressed. Following stimulation of cells *in vitro* for 48, 72 and 96 hours with 100nM CCL19, EDG1 mRNA was expressed (Figure 10B). In the presence of CCL21, HuT78 showed a slight increase in EDG1 expression, which was quickly reduced to below background levels (Figure 10B). From these results, we concluded that following a 48-96 hour exposure of HuT78 T cells to CCL19 EDG1 was up-regulated.

Expression of EDG-1 is regulated by KLF2 [118]. To examine the contribution of KLF2 to the expression of EDG-1 in HuT78 cells following stimulation with CCL19, we used western blots. Following stimulation with CCL19, KLF2 levels increased over 72 hours (Figure 11A). This increased level of expression correlated with increased expression of EDG-1. To confirm that this signaling pathway was active in primary T cells we stimulated splenic T cells with CCL19 or CCL21 (Figure 11B), in culture for 24, 48, 72 or 96 hours. Expression of KLF2 increased in primary T cells following exposure to CCL19 but not in response to CCL21. Since

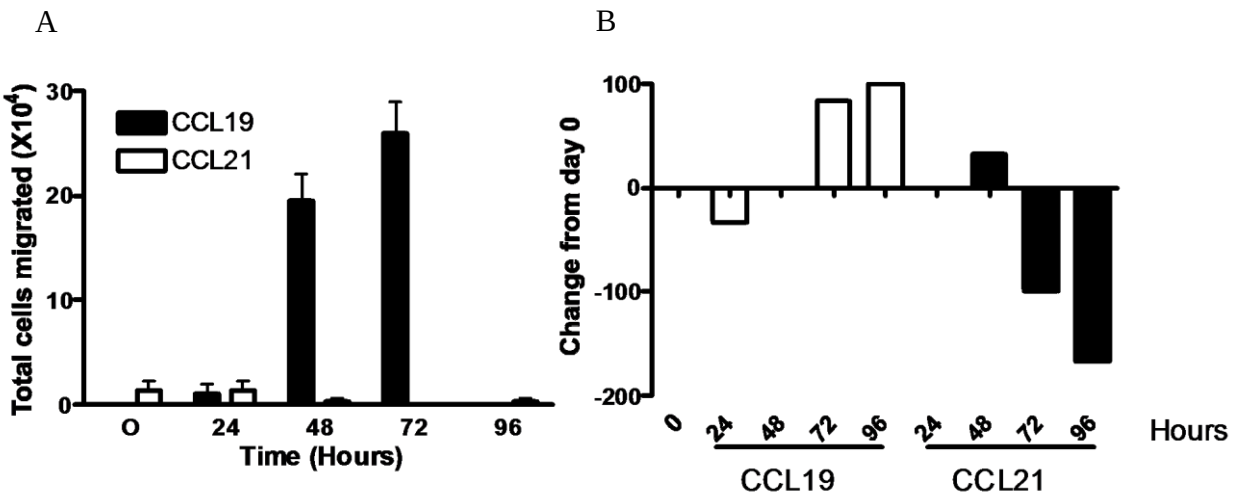

Figure 10. CCL19 stimulation of HuT78 T cells induces expression of $S1P_1$ and migration to sphingosine 1 phosphate. A) HuT78 cell migrate to sphingosine 1 phosphate after 48 and 72 hours of stimulation with 200nM CCL19 but not CCL21. B) Treatment of cells with CCL19 mediates increased levels of $S1P_1$ mRNA. Expression levels were normalized to actin.

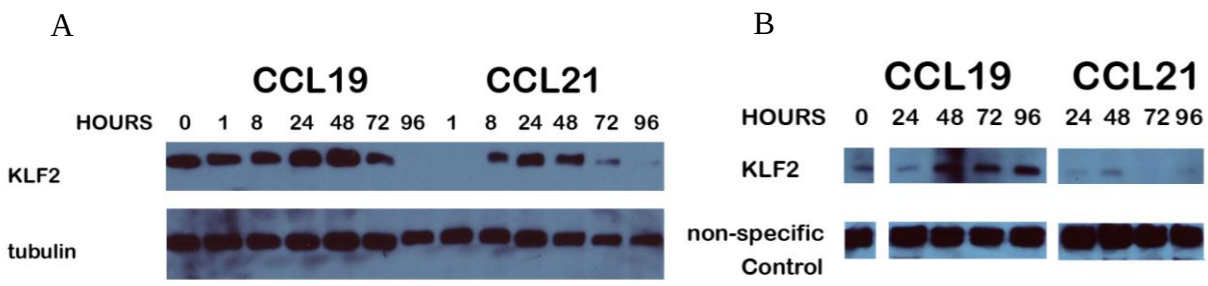

Figure 11. KLF-2 is increased in response to CCL19 treatment. A) Stimulation of HuT78 T cells with CCL19 mediates strong up-regulation of KLF2. B) Stimulation of Primary murine T lymphocytes with CCL19 mediates up-regulation of KLF-2.

KLF2 is a transcription factor for EDG-1[118], this observation demonstrated that CCL19 activation of CCR7 led to increased levels of KLF2.

Extracellular regulated kinase 5 (ERK5) has a controversial role in up-regulating the expression of KLF2 [68, 125]. To determine if ERK5 is involved in up-regulating EDG-1 following activation of T cells by CCL19, we wanted to determine if stimulation with CCL19 could mediate activation of ERK5. Activation of ERK5 can parallel activation of ERK1/2 [126, 127]. In addition, in heterologous systems, CCL19 induced a four-fold increase in activation of a ERK1/2 when compared to ERK1/2 phosphorylation levels induced by CCL21 [49]. To determine if CCL19 preferentially activated ERK1/2 in T cells that express an endogenous CCR7, we compared activation of HuT78 by CCL19 to activation by CCL21 (Figure 12A). We observed a transient increase in the phosphorylation of ERK1/2 in the presence of CCL19. In the presence of CCL21 we observed a loss of ERK1/2 phosphorylation when compared to un-stimulated cells. To confirm the signaling event in primary cells we used mouse splenic T cells (Figure 12B). We observed that while CCL21 failed to mediate ERK1/2 phosphorylation in primary murine T cells or in the HuT78 cell line, CCL19 mediated activation of ERK1/2 within 5 minutes of stimulation. While at early time-points ERK1/2 was phosphorylated, we were unable to detect activation of ERK5 during this interval (data not shown). As observed in macrophages, in T cells ERK5 runs as a 115kDa band [126]. Because up-regulation of KLF2 and the EDG1 receptor takes place over four days we examined the phosphorylation of ERK5 in the presence of CCL19, over 96 hours. After 48 hours of stimulation of HuT78 T cells with CCL19 we observed an increase in ERK5 phosphorylation, which was maintained through 96 hours of stimulation (Figure 13A). ERK5 was also phosphorylated in cells treated with CCL21, but to a lesser extent. In primary T cells, however, levels of ERK5 increased and ERK5 was

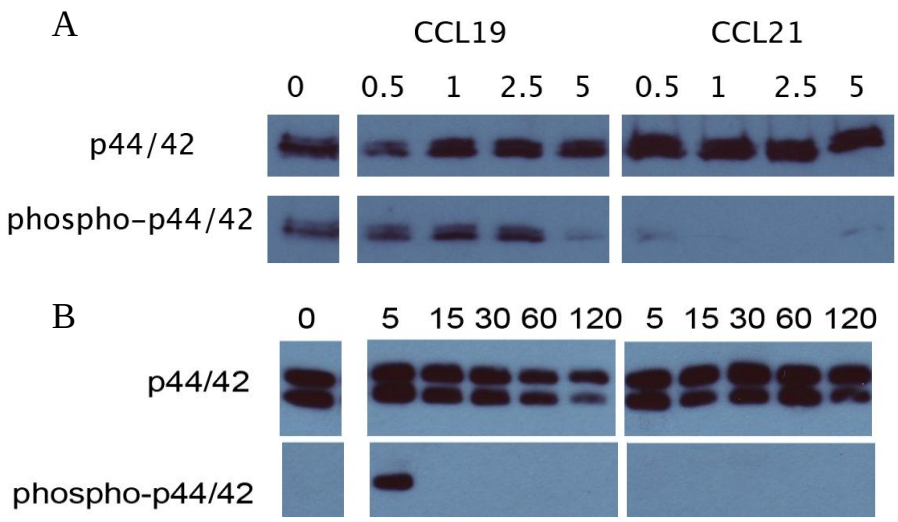

Figure 12. ERK1/2 is phosphorylated in response to CCL19. A) Stimulation of HuT78 human T cells with CCL19 but not CCL21 leads to phosphorylation of p44/42, 0-5 minutes. B) Stimulation of Hut78 human T cells with CCL19 but not CCL21 leads to phosphorylation of p44/42, 5-120 minutes.

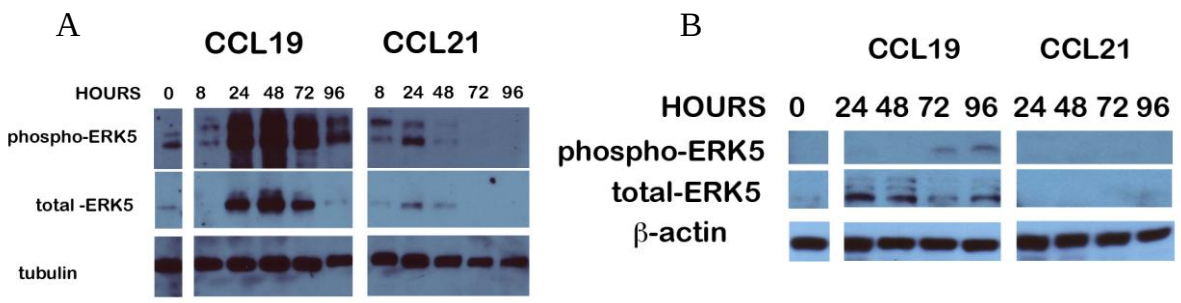

Figure 13. ERK5 is phosphorylated in response to CCL19. A) HuT78 T cell line induce ERK phosphorylation following activation with CCL19. B) Primary murine T cells induce ERK5 phosphorylation following activation with CCL19, but not CCL21.

phosphorylated only in the presence of CCL19 and not in the presence of CCL21 (Figure 13B). We concluded that in primary cells CCL19 differentially up-regulated ERK5.

4.3.3 CCL19 Expression by Activated Dendritic Cells Mediates Recruitment of Naïve T Cells

A likely source of CCL19 is mature dendritic cells, and since CCL19 mediates chemotaxis of CCR7 expressing cells, [128] to examine the contribution of CCL19 to the generation of conjugates between naïve T cells and activated dendritic cells we used an *in vitro* conjugate assay. To this end, CD11c+ bone marrow derived dendritic cells (BMDC) (Figure 14A) loaded with ovalbumin and matured in the presence of tumor necrosis factor alpha (TNFα) were incubated with naïve OTI cells, which express a transgenic T cell receptor (TCR). Under these conditions, we observed a mean of 28% T cells formed conjugates with antigen primed T cells when dendritic cells were treated with control shRNA. In contrast, in the presence of CCL19 shRNA (Figure 14B), the numbers of conjugates was significantly reduced ($p=0.030$) (Figure 14C). We concluded that CCL19 expressed by dendritic cells mediated the generation of conjugates between naïve T cells and activated dendritic cells.

4.3.4 In the Absence of ERK5 Naïve T Cells Failed to Exit the Lymph Nodes

Previous studies by other labs have used adoptive transfer assays to demonstrate that T cells have a reduced ability to migrate via CCR7 as they up-regulate EDG-1[65] in response to antigen exposure. This reduced migration is likely a result of exposure to CCL19, which rapidly internalizes ~80% of CCR7 [112, 115] since exposure to CCL21 leads to internalization of only ~25% of available CCR7. Since homozygous deletion of ERK5 is embryonic lethal [119, 129, 130] to confirm the ERK5 signaling was required to induce the EDG-1 expression following

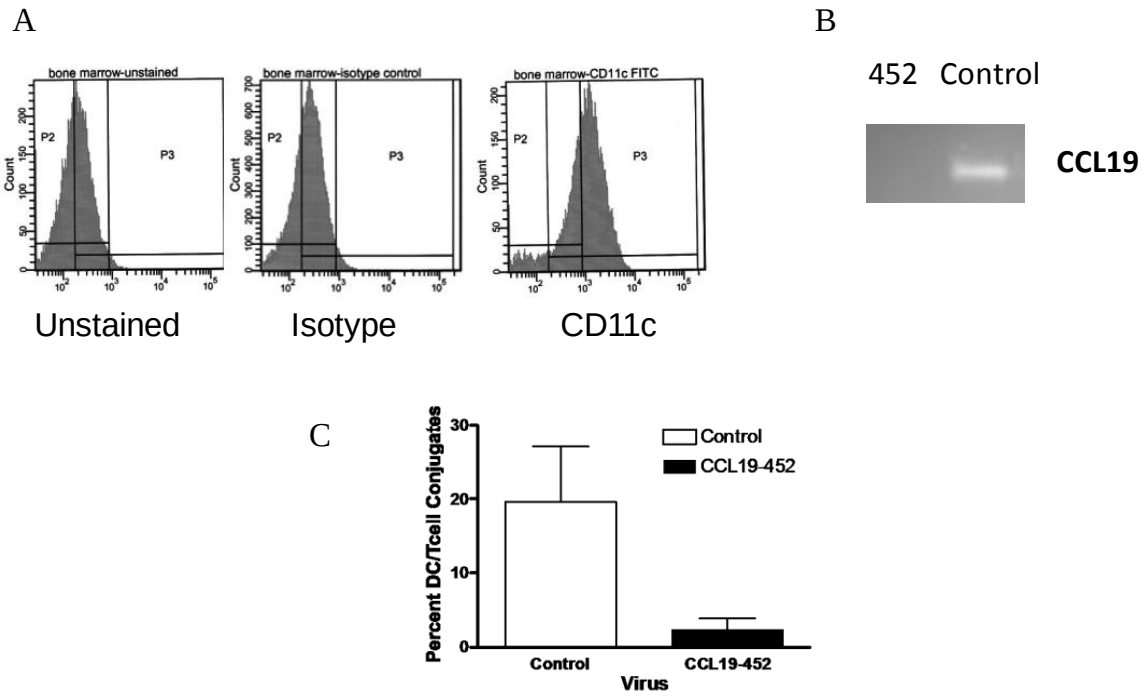

Figure 14. CCL19 knockdown results in inhibition of dendritic cell/T cell conjugate formation. A) Isolation and purification of primary murine dendritic cells indicated by expresssion of CD11c by flow cytometry. B) Confirmation of knock down of CCL19 by viral transduction. C) Loss of CCL19 expression in dendritic cells blocks T cells from forming conjugates with dendritic cells *in vitro*.

activation of T cells with CCL19, we used ERK5 specific siRNA. Splenic and lymph node T cells were nucleofected with ERK5 siRNA, cultured for 2 days, labeled with CSFE and transferred to wild type hosts. The wild type hosts were immunized with ovalbumin, and 96 hours later, adoptively transferred CSFE+ $V_{\beta 5}$+ cells were isolated by flow cytometry from draining lymph nodes and assayed for EDG-1 in a migration assay to 10nM S1P. Both OTI and OTII cells were retained in the lymph nodes (Figure 15A and 15B). Cells treated with control siRNAs expressed EDG-1 and migrated to S1P (Figure 15C). In contrast, cells treated with ERK5 siRNA failed to migrate to EDG-1. We concluded that signaling within the lymph node induces expression of EDG-1 via ERK5 and KLF2.

4.4 Discussion

The differential roles for CCL19 and CCL21 in the behavior of T cells have been elusive. In this study, we described a novel signaling pathway that differentiates signaling from CCL19 and CCL21 in T cells. The results in this paper lead to the model shown in Figure 8. We have found that CCL19/CCR7 activation mediates phosphorylation of ERK5, and in turn, up-regulation of KLF2. In response to increased levels of KLF2 the expression of EDG1 increases. Our model is supported by the lymph node distribution of CCL21 and CCL19 and the extent of internalization of CCR7 following engagement of these two ligands. We and others have shown that only ~20% of CCR7 is internalized when it is bound to CCL21, allowing for CCR7 to remain on the surface of the cell and continue to sense CCL19 or CCL21 in the lymph node. We speculate that if the T cell fails to make a conjugate with a dendritic cell that lasts for more than an hour, the signaling pathway is not turned on, allowing the T cell to

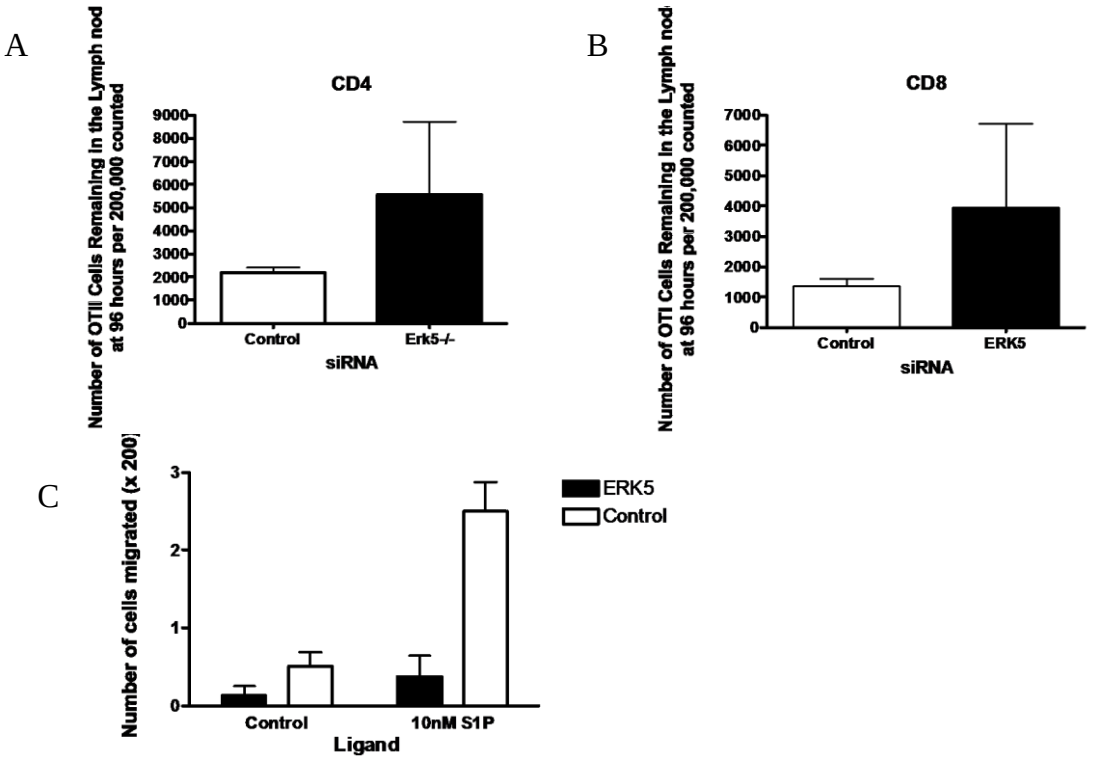

Figure 15. Loss of ERK5 results in T lymphocyte retention in lymph nodes. A) Loss of ERK5 expression prevents exit of CD4 T cells from lymph nodes. B) Loss of ERK5 expression prevents exit of CD8 T cells from lymph nodes. C) Loss of ERK5 expression prevents CCL19 treated T cells from migrating to S1P at 48 hours.

maintain its level of EDG-1 receptor and exit the lymph nodes. In contrast, if the T cell forms a conjugate with an activated dendritic cell, that expresses a cognate ligand the T cell recognizes, it maintains the close contact required to internalize over 80% of CCR7 and to activate ERK5. Studies are ongoing within our lab to determine what cytoplasmic adaptors are required for activation of ERK5.

We initiated our studies with thymic migration studies. Recent studies have reported that thymic dendritic cells regulate the evolution of regulatory T cells in the thymus [131]. These dendritic cells which are recruited from the periphery would express high levels of CCL19. Initially, CCL19 was described as a chemokine that directed the emigration of newly generated thymocytes from the thymus [108]. By *in vitro* assays, migration of dissociated thymocytes was equivalent when comparing CCL21 and CCL19. It was unclear however, why thymocytes exiting the thymus, migrated from the thymus only after an eight-hour exposure to CCL19, but not to CCL21. This delayed migration to CCL19, led to our hypothesis that transcriptional up-regulation of specific genes was required to regulate the expression of a second receptor that could be used for migration from the thymus. This implicated the existence of a second receptor that was used for emigration of cells from the thymus. Interestingly, two years later, studies examining the role of EDG-1 in thymocyte emigration, revealed EDG-1 as a possible candidate receptor.

As mentioned, CCL19 induces internalization of 80% of the CCR7 on the cell surface. In contrast, CCL21 induces internalization of ~20% of the cell surface CCR7. Therefore, it is possible that the differential signaling is due to differences in levels of CCR7 on the surface. Such a hypothesis would be difficult to test without generating numerous T cell lines that express different levels of CCR7 on the cell surface. An alternative explanation is that internalization of

CCR7 via CCL19 vs. CCL21 mediates recruitment of different adaptor proteins that remain associated with the cytoplasmic face of the receptor following recycling of the receptor. We have observed that internalization of CCL19 bound CCR7 mediates recruitment of arrestin 3, while CCL21 does not. Since CCL19 mediates internalization of CCR7 through arrestins, while CCL21 does not[112], it is possible that the activated form of CCR7 recruits ERK5 following engagement of CCL19 via arrestins. The recruitment of arrestins takes place within 5 minutes of CCR7 activation by CCL19, and while ERK1/2 is activated within this timeframe, activation of ERK5, requires a minimum of one hour of exposure to CCL19. From this observation, we speculate that other adaptors may be required, that associate only after CCR7/CCL19 has trafficked within the cell to sites that allow for recruitment of these adaptors. The adaptors would then remain associated when the receptor recycles. In contrast, signaling through CCR7/CCL21 fails to activate this pathway since it takes a different pathway when it is internalized [46, 49, 112, 114]. If the receptor is re-internalized rapidly, due to the presence of ligand the subsequent internalizations may recruit novel adaptors, to the already modified cytoplasmic tail. At some point ERK5 associates with the activated receptor and is internalized. CCR7 has been observed in the nucleus in some cells [71]. A function for CCR7 in the nucleus, however has not yet been defined.

Since the $ERK5^{-/-}$ mouse is embryonic lethal, we were unable to use the T cells from that mouse to study ERK5 signaling in the absence of ERK5. Therefore, we used ERK5 siRNA to reduce expression of ERK5 in primary T cells. This allowed us to examine signaling via CCR7/CCL19 in the absence of ERK5. From our studies, we concluded that ERK5 is indeed required for up-regulation of EDG-1. These results are supported by reports in which a CCL19 specific antagonist was used to block signaling through CCR7/CCL19. In this study, following

pre-treatment of mice with the CCL19 antagonist $ELC_{8\text{-}83,}$ the mice were injected with T cells expressing the ovalbumin TCR, and stimulated with ovalbumin. In support of our studies, $ELC_{8\text{-}83}$ prevented egress of T cells from the lymph nodes. More recently, T cells adoptively transferred to a $CCL19^{-/-}$ mouse, failed to leave the lymph nodes [34]. In these studies and in our studies, loss of signaling through CCR7/CCL19 blocked egress of T cells from the lymph nodes.

KLF2 expression is tightly regulated in T cells during differentiation and maturation. Over-expression of activated ERK5 leads to up-regulation of KLF2 in a reconstituted fetal thymic organ culture, while dominant negative ERK5 blocks KLF2 [119] . Recently it was reported that Foxo1, one of four members of the Foxo subfamily of transcription factors, that control life span cell cycle progression and [132, 133] apoptosis, controls the expression of CCR7 and KLF2[133]. In these studies, in response to homozygous deletion of a floxed Foxo1, in a CRE/lox mouse, the levels of CCR7 were reduced along with the levels of KLF2. In other studies, using a microarray of RNA's enriched for immune function, KLF2 mRNA levels were found to be up-regulated during positive selection at the same time when CCR7 is first expressed in T cells [134] in the thymus and lymph nodes. This was not surprising, given the role that CCR7 has in the up-regulation of KLF2. Furthermore, KLF2 levels increase following *in vitro* stimulation of naïve T cells, with IL-2 or IL-17 [135]. KLF2 expression correlated with long term survival.

ERK5 activation has been associated with Src activation and the increased migration of fibroblasts by mediating changes in the actin cytoskeleton, in particular mediating a loss of stress fibers [136, 137]. In addition, activation of ERK5 in fibroblasts promotes invasive phenotype of the cells and degradation of ECM [136]. In fibroblasts, transformed by activated c-Src or v-Src, ERK5 mediates podosome formation and the invasive behavior of cells, by regulating the

induction of matrix degradation. Since mature T cells leave the lymph nodes, and extravasate to sites of infection, it is questionable whether the observed change in ERK5 activation, reflects increased migratory potential. In addition, we observed a decrease in the level of ERK1/2 phosphorylation. Since the levels of ERK5 phosphorylation are below our limits of detection, by western blot, it is unclear if along with pERK1/2, ERK5 phosphorylation levels decrease following exposure to CCL21. Such a decrease could slow matrix degradation and extravasation of cells into the tissues. We observed an increase in the level of EDG-1 expressed in T cells over the 96 hour CCL19 stimulation period making it difficult to determine if there were any changes in the overall migration potential of the cells to EDG-1 over the time course. At present, studies are underway in our laboratory to examine the role of ERK5 in regulating migration in CCL19 activated T cells to chemokines other than CCL19 and CCL21.

ERK5 plays roles in cell proliferation, survival and differentiation [126, 138-144]. In a macrophage cell line, and in primary human macrophages ERK5 phosphorylation in response to signaling through the CSF-1 receptor, mediates proliferation [126]. This proliferation is linked to signaling through c-jun, which correlates with translocation of ERK5 into the nucleus. Our studies measured ERK5 phosphorylation in the presence of 40nM CCL19 or CCL21. T cells that lacked expression of CCR7 had reduced proliferation [145]. It is tempting to speculate that a possible role for activation of ERK5 in response to a CCL19 rich dendritic cell, would be to help to promote proliferation of the activated T cell.

In conclusion, our data defines differential signaling events that take place within naïve T cells following activation by CCL19 or CCL21. This signaling information may be used to combat a variety of diseases. That $CCL19_{8\text{-}83}$ and m-SLC4 are CCL19 and CCL21 specific antagonists, which block lymph node entry and dendritic cell/T cell association, respectively,

may lead to the identification of pharmaceutical agents that specifically block lymph node entry or sampling of dendritic cells/exit from the lymph nodes. Such therapies could be important in treating autoimmune diseases, lymphoproliferative disorders or asthmas that have been linked to CCR7 [146-152].

4.5 Experimental Procedures

4.5.1 Cell Lines and Mice

The HuT78 human T cell line was purchased from ATCC and maintained in HuT78 media (RPMI 1640 (Invitrogen)/10% heat inactivated fetal bovine serum (Hyclone) /2nM L-glutamine (Invitrogen)) in a humidified atmosphere at 37°C and 5% CO_2. C57BL/6 mice were purchased from Jackson Labs. C57BL/6-Tg(OT-I)-recombination-activating gene 1 deficient *(RagI$^{-/-}$)* mice were purchased from Taconic. T lymphocytes isolated from OT-II.2a/RAG1 C57BL/6 were maintained for up to one week in T cell media (RPMI 1640, 10% heat inactivated fetal bovine serum, 2nM L-glutamine, 50µM β-mercaptoethanol, 20U/ml IL-2, 100U/ml penicillin/100µg/ml streptomycin).

4.5.2 Fetal Thymic Organ Culture

Thymic organ cultures were prepared as described [122], with the following modifications. Thymic lobes were removed from C57BL/6 fetuses at 15.5 days postcoitum, cultured at the air/media interface on 3µm^2 transwell filter membranes in a humidified 5%CO_2/air incubator at 37°C. Cultures were maintained in FTOC media ((RPMI1640 (Sigma)/10% heat inactivated fetal bovine serum (Hyclone) /50µM β-mercaptoethanol (Fisher),

2mM L-glutamine (Sigma), 1 x nonessential amino acids (Cellgro), 10mM HEPES (Cellgro), 1mM sodium pyruvate (Cellgro), 100 U/ml penicillin (Cellgro), and 100µg/ml streptomycin (Cellgro)), for 5 days to allow for the development of thymocytes to a broad range of developmental stages that resemble the distributions of an adult thymus [122]. Thymic lobes were washed, placed on a fresh 3µm^2 transwells (Millipore) for 2 days after adding 100nM of either murine CCL19 (R&D) or murine CCL21 (R&D) or FTY720 (Cayman). The number of cells that migrated to the lower wells were counted by hemacytometer.

4.5.3 Chemotaxis Assays

Chemotaxis assays were carried out as described[112]. For chemotaxis assays, Hut78 cells were treated with 40 nM murine CCL19 (R&D), CCL21 (R&D) or an equal volume of vehicle (phosphate buffered saline (PBS)) for 96, 72, 48 and 24 hours and migrated across a 5um membrane (Neuro Probe, Inc.) at 37°C and 5% CO_2 for 2 hours to 10nM S1P (Sigma) in serum free RPMI 1640 medium in a 48 well chemotaxis chamber. Cells in the bottom chamber were counted by hemacytometer. Assays were performed in duplicate and replicated 3 times.

4.5.4 Dendritic Cell/T Cell Conjugates

Bone marrow derived dendritic cells were generated from C57BL/6 wildtype mice as described[153]. Briefly, 2 x 10^6 cells isolated from bone marrow were plated on bacterial petri dishes and cultured in 10 ml DC media (RPMI 1640, 10% heat inactivated fetal bovine serum, 100µg/ml penicillin-streptomycin, 50µM β-mercaptoethanol, 20ng/ml murine granulocyte macrophage colony stimulating factor (GM-CSF) (R&D), and 2nM L-glutamine). At 48 hours (Day3), 10ml of fresh DC media was added to each dish. On days 6 and 8, 10ml of

supernatant/cells was removed, cells isolated by centrifugation (90 x *g*) and resuspended with fresh DC medium. On day 9 non-adherent cells were collected and nucleofected using the Amax DC Nucleofection kit, Nuclefector II, and program Y-001 (Amaxa) with shRNA against CCL19 (Origene). 10^6 nucleofected cells were plated into each well of a 12 well dish and RNA knocked down for 48 hours. On day 11 immature dendritic cells were labeled with (5uM) 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) for 10 minutes at 37°C, washed, primed with 50ug/ml ovalbumin, and matured with 100ng/ml TNFα. T cells were isolated from OT-II2a/Rag1C57BL/6 spleens by passing spleens through a wire mesh. T cells were purified using a negative selection, mouse T cell enrichment kit according to manufacturers' directions (EasySep). Purified T cells were labeled with (10uM) (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) CMTMR for 30 minutes at 37°C, washed and plated in the well with the ovalubumin-primed, CFSE labeled mature dendritic cells. Cells were allowed for form conjugates for two hours, fixed and conjugates counted per 100 dendritic cells.

4.5.5 Western Blots

HuT78 and primary cells were counted by hemacytometer or following migration assays, whole thymic organ cultures were lysed in radio immunoprecipitation assay buffer (RIPA) (100mM Tris pH 8.0, 300mM NaCl, 2% Nonidet P40, 1% sodium deoxycholate, 0.2% SDS) supplemented with protease inhibitor cocktail (Sigma), 1mM sodium ortho-vanadate, 1mM sodium pyrophosphate, 10mM sodium fluoride (Sigma) and 1mM β-glycerophosphate (Sigma). Lysates were centrifuged for 20 minutes following homogenization. 5 x 10^5 cell equivalents were solubilized in sample buffer with 10 mM dithiothreitol, fractionated on 10% SDS poly

acrylamide gels and transferred to PVDF membranes. Membranes were incubated in 5% milk/Tris buffered saline (50mM Tris-HCl pH→7.5, 150mM NaCl) 0.1% Tween-20 (TBST) and probed with anti-ERK5 (Cell Signaling Technologies), anti-α-tubulin (Cell Signaling Technologies), anti-β-actin (Cell Signaling Technologies), anti-KLF-2 (CeMines) or anti-MIP3β (AbCam). To detect phospho-ERK5, membranes were incubated in 3% bovine serum albumin (Sigma #A3058) and probed with anti-phosphoERK5 (Upstate). MIP3β was detected with HRP rabbit anti-goat IgG and all other antibodies were detected with HRP goat anti-rabbit IgG (Pierce) and immune complexes were developed with chemilluminence Supersignal West Femto (Pierce). Protein bands were visualized with X-MAT film and quantified with a Fuji LAS-4000. Membranes were stripped using Blotfresh (Signagen) stripping buffer for 10 minutes (SignaGen), rinsed three times in TBST, blocked and re-probed.

4.5.6 Reverse Transcriptase-PCR

Total RNA was extracted from Hut78 cells or splenocytes using TRIzol (Invitrogen). cDNA was prepared as follows: 50ng random primers (Invitrogen), 1µg RNA, 0.25mM dNTP (TakaRa) and ddH_2O, were heated to 65°C for 5 minutes and chilled on ice. 5X First Strand Buffer (Invitrogen) was added to the mixture and adjusted to 1X in the presence of 5 mM DTT (Invitrogen), and 40 U RNAse (Promega). The reaction was incubated at 25°C for 2 minutes, and 200U Super Script II™ RT (Invitrogen) was added. The cDNA was primed at 25°C for 10 minutes, and then extended by incubating at 42°C for 50 minutes. Super Script II™ RT was inactivated by heating to 70°C for 15 minutes. To assay for expression, PCR was carried out by addition of 1x Green Go Taq flexi buffer (Promega), 1.5mM $MgCl_2$ (Invitrogen), 0.25mM dNTP (TakaRa), 1U Taq DNA polymerase (Invitrogen), cDNA, H2O and mRNA specific primers.

Primers used: murine $S1P_1$ [65] and murine β-actin forward: 5'ATGACGATATCGCTGCGCTG3' reverse: 5'AGTAACAGTCCGCCTAGAAG3'. PCR products were resolved on a 2% agarose gel containing ethidium bromide. Products were amplified 30 cycles using a thermocycler. $S1P_1$ and β-actin yielded products of the predicted size.

4.5.7 Adoptive Transfer

Adoptive transfers were carried out as described[65], with the following modifications. OT-II.2a/RAG1 C57BL/6 splenocytes were isolated and 2 x 10^7 cells were Nucleofected (Amaxa) with ERK5siRNA or control siRNA (Ambion) in OptiMEM. Endogenous ERK5 was knocked down for 72 hours, and labeled with 1μM 6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular probes)for 20 minutes at 37°C, rinsed and transferred intravenously to C57BL/6 recipients. The day after transfer, (day 0), mice were immunized subcutaneously with a total of 200μg ovalbumin (Sigma) emulsified in complete Freund's adjuvant (Sigma) (ratio 1:1) at 4 sites along the back. After 96 hours, mice were euthanized, and lymph nodes were analyzed for migration to sphingosine 1 phosphate, CCL19, CCL21 and for numbers of CSFE+ cells in lymph nodes, by flow cytometry and hemacytometer. All assays were performed in duplicate for sphingosine 1 phosphate or chemokine and were repeated using cells from a minimum of two different animals of each type.

CHAPTER 5 – EXPRESSION OF A CHEMOKINE RECEPTOR, CCR7, MEDIATES METASTASIS OF BREAST CANCER TO THE LYMPH NODES AND REDUCES METASTASIS TO THE LUNGS IN MICE

5.1 Abstract

The C-C chemokine receptor 7 mediates lymphocyte migration to lymph nodes in response to its ligand CCL21, where it regulates lymph node organization and proliferation. While CCR7 has been implicated in lymphocyte migration to lymph nodes, the molecular mechanism that mediates targeting of breast cancer metastasis to the lymph nodes has yet to be defined. To explore the mechanisms used by breast cancer to migrate to the lymph nodes, we used the MMTV-PyVmT mammary tumor cells (PyVmT). *In vitro* we found that expression of CCR7 controlled migration of PyVmT mouse mammary tumor cell migration to CCR7 ligands as well as MDA-MB-231, MCF-7 and T47D human breast tumor cells. To define a physiological significance for CCR7 regulation of migration, we used the PyVmT transgenic mouse model of metastatic breast cancer. When PyVmT cells transfected with control vector were orthotopically transferred to the mammary fat pad of FVB mice, there were significantly more lung metastasis (10/10 mice) but not to the lymph nodes (0/10), which repeated the known phenotype. In contrast, CCR7-transfected PyVmT (PyVmT-CCR7) cells transplanted to the fat pad metastasized to the lymph nodes (6/10 mice) but had a reduced rate of metastasis to the lungs (4/10 mice). These data show, for the first time, that expression of CCR7 in breast cancer

can target migration of breast cancer cells to the lymph nodes, and in the future may be used as a tool for studying lymph node.

5.2 Introduction

When a breast tumor has metastasized to more than four lymph nodes, a woman's chance of surviving breast cancer is significantly reduced. Consequently, clinical staging of breast cancer involves determining whether breast tumors have metastasized to the lymph nodes. It is puzzling however, that subsets of these women are long-term breast cancer survivors, even when breast cancer metastases have been detected within their lymph nodes. A clue to the differences in survival rates among women may have been revealed when it was observed that in patients with C-C chemokine receptor 7 (CCR7) expressing breast tumors that had metastasized to the lymph nodes, tumor metastases were not found in the lungs, liver or brain, but instead were restricted to the surgically removable lymphoid organs [154].

CCR7 is activated by binding either CCL19 or CCL21 [155, 156]. Under normal circumstances, naïve T cells use CCL21 to travel to and enter lymphoid tissues from the blood via high endothelial venules (HEV) and migrate through the T cell zone before returning to the circulation. Naive T cells are activated when they migrate toward and interact with activated CCL19-expressing dendritic cells, then bind to the appropriate major histocompatibility/antigen complex[157]. CCL19 and CCL21 chemokines have important roles in establishing and maintaining the architecture [158] of the secondary lymphoid organs, where they coordinate the adaptive immune responses between dendritic cells, B-cells and T cells. Thus, chemokines are

critical mediators of the inflammatory response and may contribute to tumor cell lymph node localization in CCR7 expressing tumors.

At present, it is thought that metastasis results from non-random events, where tumor cells target specific organs in response to unique factors found within the targeted organ [159]. These factors control tumor cell adhesion, migration and organ invasion. Small (8-12KDa) chemotactic cytokines, termed chemokines have been recently identified as contributing to the metastatic behavior of tumor cells [72], but the specific functions regulated by chemokines like CCL19 and CCL21 that contribute to breast tumor metastasis have remained elusive. During metastasis, a tumor cell must detach from the primary tumor, extravasate to the micro vessel walls, and migrate to the target tissue. CCR7 regulates lymphocyte adhesion, migration and proliferation, and therefore is an important receptor to understand in the regulation of tumor metastasis.

CCR7 is up regulated in certain breast cancers [72] and is associated with lymph node metastases [71]. *In vitro* MDA-MB-231 and MDA-MB-361 breast cancer cell lines and a primary sample from a patient were capable of migrating to CCL21. To date, it remains unclear, whether CCR7 can promote migration of breast cancer from the mammary fat pad to the lymph nodes. We have adapted a mouse mammary tumor virus (MMTV)-PyVmT model of metastatic breast cancer, which selectively metastasizes to the lungs, to express CCR7 in the PyVmT cells to allow us to determine the role for CCR7 in breast cancer progression and metastasis. We have observed that CCR7 directs the migration of metastasis to the lymph nodes, instead of the lung where PyVmT cells that lack CCR7 expression metastasize. In addition, we have found that similar to its role in lymphocytes, CCR7 alters the differentiation state of PyVmT cells as reflected in their increased proliferation rate of 3 D mammospheres *in vitro*.

5.3 Results

5.3.1 CCR7 is Expressed on Human Primary Cells

CCR7 is up-regulated on the surface or in the cytoplasm of cells histochemical preparations of tumors [71, 72, 154, 160, 161]. To determine whether CCR7 is expressed on the surface of breast cancer cells, we examined the expression levels of CCR7 in isolated epithelial cells by surface labeling with anti-CCR7 antibodies (n=5) and quantified by flow cytometry. To this end primary cells were dissociated with collagenase and isolated by positive selection using magnetic beads that target epithelial cell markers. We found that CCR7 was elevated on freshly isolated primary cells and CCR7 receptor was expressed in malignant breast epithelia as compared to normal. Even this difference in receptor expression levels can profoundly affect the cellular response to ligand binding [162].

5.3.2 Generation of CCR7 Expressing PyVmT Cells

We hypothesized that CCR7 is used by breast cancer to metastasize to the lymph nodes, a process that requires adhesion, cell spreading and migration. To examine the effect of low levels of CCR7 on breast cancer cell adhesion, spreading and migration, we examined the effects of stimulating CCR7 in a murine breast cancer cell line PyVmT [158], which lacks endogenous CCR7 as determined by RT-PCR (data not shown). To facilitate detection of CCR7 in PyVmT tumors *in vitro* we used a FLAG epitope fused to the N-terminus of CCR7 to generate transient transfectants that expressed N-FLAG-CCR7 (PyVmT-CCR7). Transient transfection of the PyVmT cells resulted in a modest increase in the levels of murine CCR7 expression that were

similar to levels that we have observed in fresh tumor isolates from human breast cancer patients and (Figure 16).

5.3.3 CCR7CCL19 Promotes Spreading of PyVmT Cells on Fibronectin

Adhesion of tumor cells, via β1 integrins promotes tumor metastasis [85]. To determine if CCR7 expression affects cell adhesion, spreading or migration via β1 integrins we used *in vitro* assays. Coverslips were coated with the 10μg/ml β1 integrin ligand fibronectin, and cells were allowed to adhere for 5 minutes or spread for an hour. To confirm specificity for the β1 integrins, we used a β1 integrin, function blocking antibody. Activation of CCR7 on PyVmT-CCR7 cells had no effect on cell adhesion via the β1 integrins (Figure 17A). In contrast, we observed that CCR7 promoted β1 integrin-specific cell spreading, in the presence of CCL19, a ligand for CCR7(Figure 17B).

5.3.4 CCR7 Promotes Migration of Breast Cancer Cells to CCL19 and CCL21

Following detachment and extravasation, an essential step in metastasis is migration of cells from the primary tumor to the site where the metastasis attaches and grows. To determine if CCR7 can promote migration to CCR7 ligands, we used an *in vitro* transwell chemotaxis assay on β1 integrin coated surfaces. We found that CCR7 preferentially promoted migration to CCL19 and CCL21 under these conditions (Figure 18A and 18B). From these results we conclude that CCR7 can promote directed migration of cells to lymphoid organs in response to CCL19 and to CCL21.

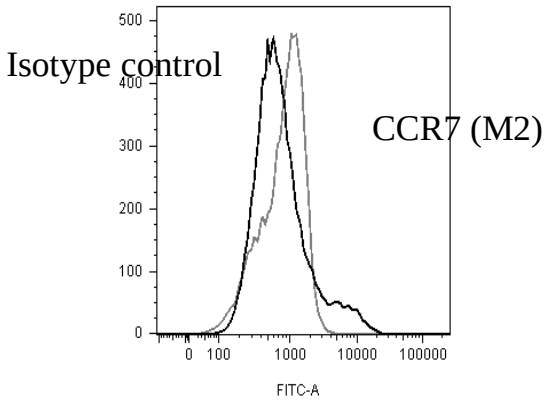

Figure 16. Transient transfection of PyVmT with N-FLAG-CCR7 results in low level of CCR7 expression. PyVmT were transiently transfected with a N-terminally tagged murine CCR7, dissociated from culture and assayed for expression of FLAG with an M2 antibody, counterstained with FITC-conjugated, donkey anti-mouse.

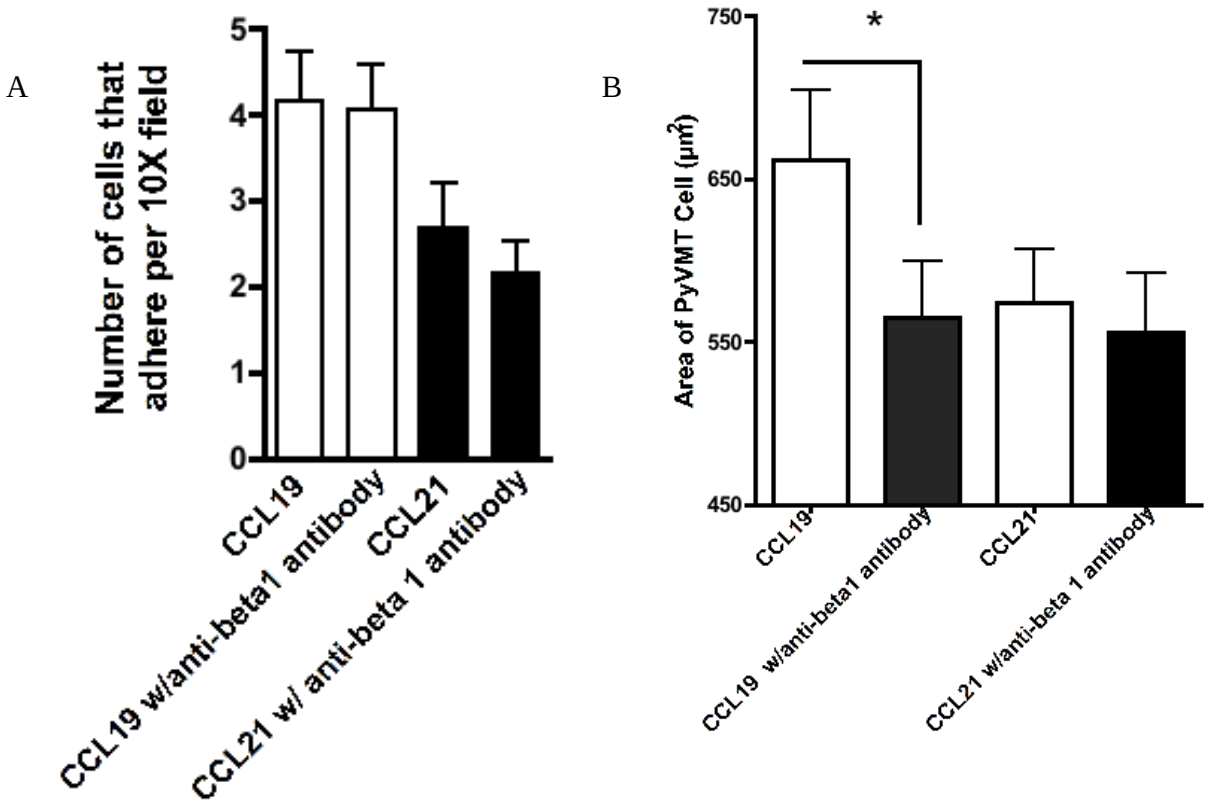

Figure 17. CCR7 activation by CCL19 promotes spreading but not adhesion. A) PyVmT cells were seeded onto fibronectin incubated coverslips in the presence of CCL19 and CCL21 +/- anti-β1 antibody and allowed to adhere. Number of cells were counted per 10X field. B) PyVmT cells were seeded onto fibronectin incubated coverslips in the presence of CCL19 and CCL21 +/- anti-β1 antibody and allowed to spread for 1 hour. Cell surface area was measured.

5.3.5 Development of PyVmT/FVB Mouse Model of Breast Cancer

To examine the physiological significance of our *in vitro* observations, we used the PyVmT model [158], that has been shown to form tumors analogous to stage IV human breast cancer, making this an ideal model to study breast cancer progression. The PyVmT tumor cells that were isolated had been selected for metastasis primarily to the lungs [158]. In the original study, 500,000 PyVmT tumor cells were implanted into the fifth mammary fat pad of syngeneic FVB mice and within forty two days, 100% of the mice uniformly developed tumors that metastasized only to the lung [158]. Previous studies, which examined the role of the CXCR4 receptor in metastatic behavior of tumor cells, revealed that differences were observed in outcome between CXCR4 expressing tumors and vector controls only when low levels of tumor cells were seeded, [163]. Therefore, to determine the lowest number of PyVmT cells that could be used to generate tumors in the FVB mice, we compared the numbers of tumors generated following the introduction of 10^6, 10^5 and 10^4 tumor cells. In our studies 100% (6/6) of the animals with 10^6 tumor cells developed palpable tumors within 24 days, 100% (6/6) of the animals with 10^5 tumor cells developed tumors within 50 days, and 33% (2/6) of the mice developed tumors when 10^4 cells were implanted (Table 1) .

Therefore, we injected 10^5 PyVmT-CCR7 or 10^5 PyVmT-vector (CCR7 (-)) tumor cells into the fifth mammary fat pad of the FVB mice. To obtain statistically significant data, we used 10 mice per group. Calipers were used to measure tumor length, width and height. Tumor volumes were calculated as a product of these three measurements. Volumes of the

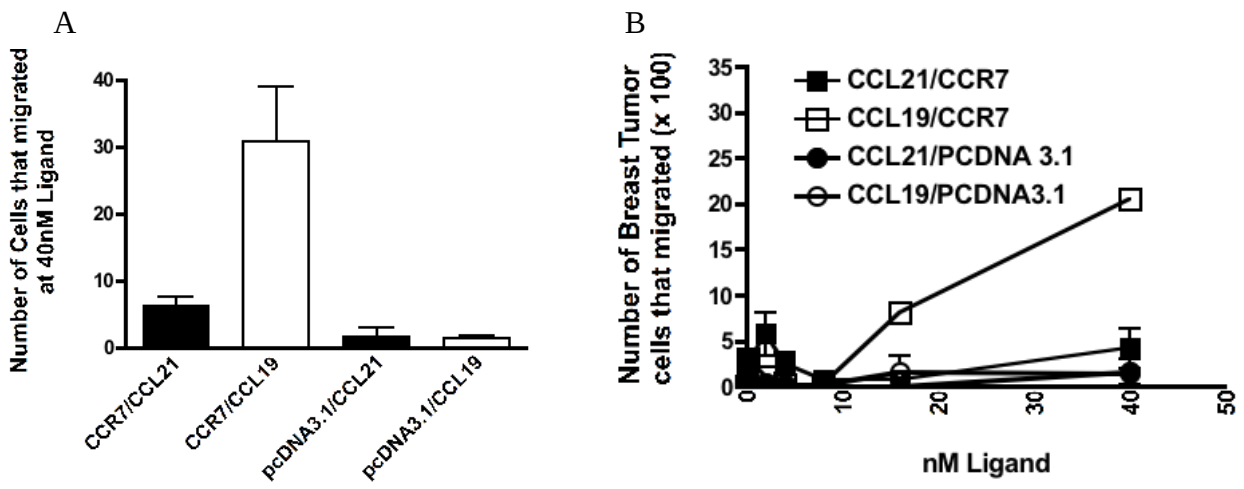

Figure 18. Stimulation of PyVMT-CCR7 cells with 40nM CCL19 or 40nM CCL21 promotes migration when compared to PyVMT-pcDNA3.1 controls. A) Graphical representation of PyVmT migration to CCL19 or CCL21 on fibronectin-coated membranes (10μg/ml). After 3 hours migrated cells were counted. (n=3) B) PyVmT transfectants were allowed to migrate to 50, 15, 7.5 and 3.75 nM ligand for 3 hours on fibronectin (10μg/ml) coated membranes. After 3 hours migrated cells in the bottom well of the chemotaxis chamber were counted (n=3).

Number of Cells Implanted	Average Days to Develop Tumor	Number of Animals with Tumors
1,000,000	24	6/6
100,000	24	6/6
10,000	29	2/6

Table 1. Number of PyVmT cells implanted results in number of animals that develop tumors.

PyVmT-CCR7 tumors were significantly greater than the tumor volumes observed in the PyVmT-vector control tumors over the course of the study (Figure 19A and 19B). On day 48 post-implantation, tumors in two of the PyVmT-CCR7 mice grew to sizes that were greater than 20mm in a single dimension, our criterion for euthanization, and were therefore immediately removed from the study.

5.3.6 CCR7 Expression Results in Lymph Node Metastasis and Decreased Lung Metastasis

To determine if CCR7 altered the metastatic behavior of the tumors, we used RT-PCR to determine if PyVmT cells were present in the lymph nodes. To this end, we isolated the draining lymph node, and used half of the lymph node to isolate mRNA for PCR and used the other half for histological preparations. By PCR we found that 60% of the PyVmT-CCR7 animals developed metastases in the draining lymph nodes. In contrast, 0% of the PyVmT-vector controls had metastases within their lymph nodes. Additionally, we found that while four of the PyVmT-CCR7 animals developed metastasis in their lungs, 100% of the PyVmT-vector control mice had between one and four metastases in their lungs (Table 2). It is important to note that, on blind evaluation of the remaining lymph node tissue, we found that the lymph nodes of the mice bearing the PyVmT-CCR7 tumors were dysmorphic and had few if any clear germinal centers, when compared to the lymph nodes of the PyVmT-vector control mice, which had clear germinal centers and normal morphology (Figure 20).

5.3.7 CCR7 Promotes Increased Growth in Response to CCL19 and CCL21 in 3D Cultures

To define the mechanism that mediated differences in growth between PyVmT-CCR7 and PyVmT-vector control animals, we used 2D tissue culture on a flat tissue culture surface.

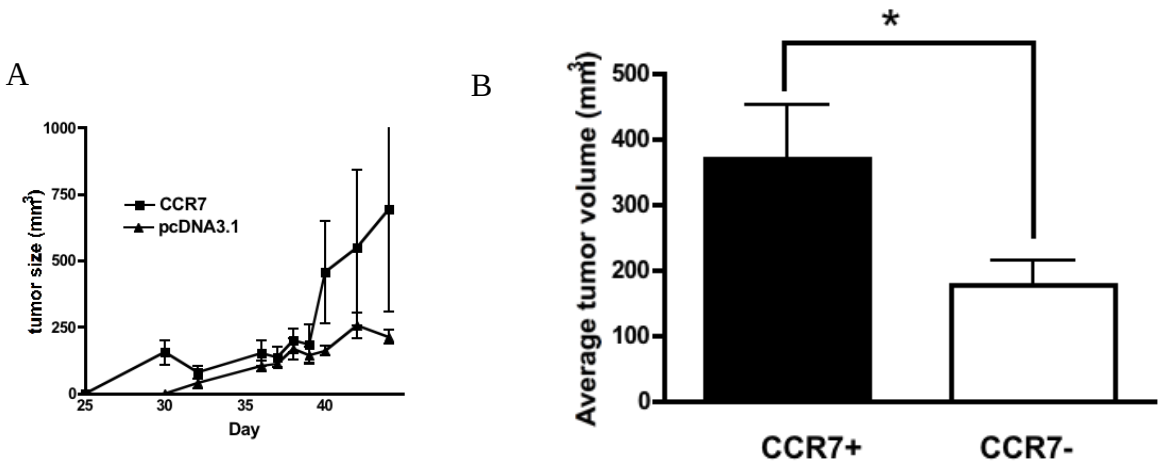

Figure 19. Average tumor volume of CCR7 (+) tumors was significantly greater than CCR7 (-) tumors on day 48 A) 105 cells were injected into the fifth mammary fat pad of FVB mice. Palpable tumors were detected as early as day 25 in both groups. Tumors volume as measured with calipers (length x width x height) was significantly greater in the PyVmT-CCR7 mice when compared to the PyVmT-vector controls p=0.002. B) At day 48 the average tumor volume of PyVmT-CCR7 tumors (259.9±71.22) was significantly larger than the PyVmT-vector controls (119.1± 27.30).

Observed differences in mice injected orthotopically with PyVmT-CCR7 or PyVmT-vector control cells		
Cell Line	Animals with Tumors in Lung (average number of tumors)	Presence of PyVmT in lymph node by PCR
PyVmT-CCR7	4/10	6/10
PyVmT-Vector	10/10	0/10

Table 2. CCR7 promote metastasis of PyVmT cells to the lymph nodes and decreases metastasis to the lungs.

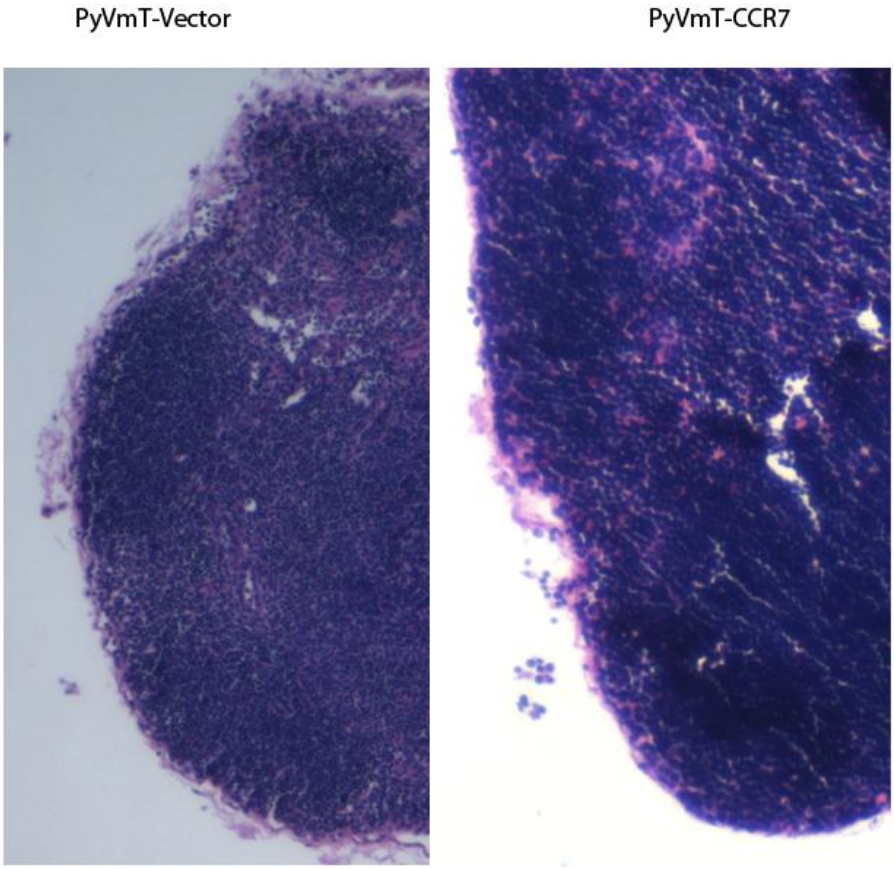

Figure 20. CCR7 disrupts the architecture of lymph nodes. H&E stained histological sections from PyVmT CCR7+ mice and PyVmT Vector control mice.

We found that when cells were grown on flat surfaces, there was no statistically significant difference in the rates of proliferation of CCR7 expressing cells in the presence of CCR7 ligands CCL19 and CCL21 (Figure 21A). Indeed, cells grown in the absence of ligand grew marginally better than cells grown in the presence of CCL19 or CCL21.

It is well recognized that growth of tumor cells in three-dimensional (3D) serum free mammosphere culture leads to growth of different lineages of epithelial cells [164]. As mentioned, CCR7 mediates differentiation of T lymphocytes. Therefore, to determine if CCR7 promoted growth under conditions that allows differential proliferation of PyVmT we used a mammosphere assay. To this end, we allowed PyVmT-CCR7 or PyVmT-vector cells to proliferate for seven to ten days in the presence of CCL21 or CCL19 in mammosphere media [165]. We observed a significant difference in the size of PyVmT-CCR7 mammospheres when compared to PyVmT-vector control mammopsheres (Figure 21B and 21C) and this growth was inhibited by blocking β1 integrins in human MCF-7 cells (Figure 22). These results support the increased growth rate of the PyVmT-CCR7 that we observed in our FVB mice *in vivo*. From these studies we conclude that CCR7 can regulate the size of tumors as well.

5.4 Discussion

In patients with CCR7-expressing breast tumors that had metastasized to the lymph nodes, tumor metastases were not found in the lungs, liver or brain, but instead were restricted to the surgically removable lymphoid organs [154]. Women with CCR7+ metastases in their lymphoid organs do not exhibit metastasis to vital organs. Without an animal model, it has

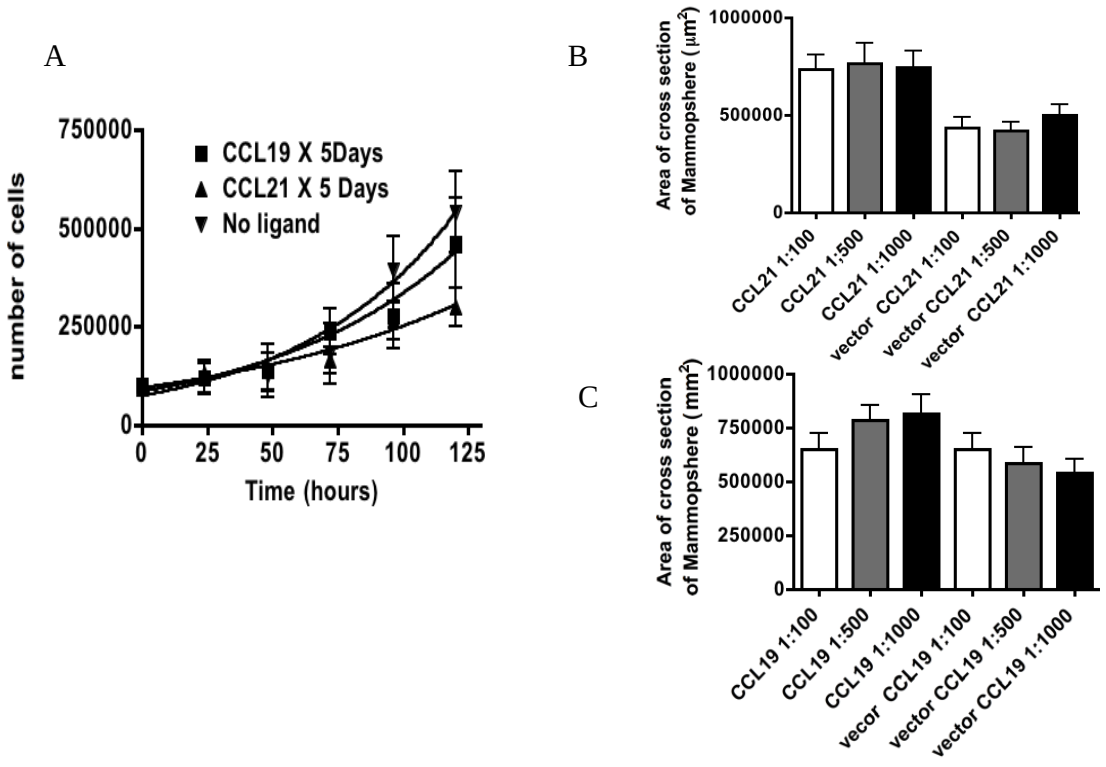

Figure 21. Expression of CCR7 affects mammosphere size in 3D culture but has no effect on mammosphere number. A) 10^5 PyVMT-CCR7 cells were allowed to grow ± ligand in 2D tissue culture for 5 days. Cells were trypsinized and counted, in duplicate. Results are means ± SD. (n=3) B) PyVmT-CCR7 and PyVmT-vector control cells were allowed to grow into mammospheres in low adhesion plates in the presence of CCL21. C) PyVmT-CCR7 and PyVmT-vector control cells were allowed to grow in mammosphere media in low adhesion plates in the presence of CCL19. Numbers of mammospheres were counted and sizes measured. (n=2)

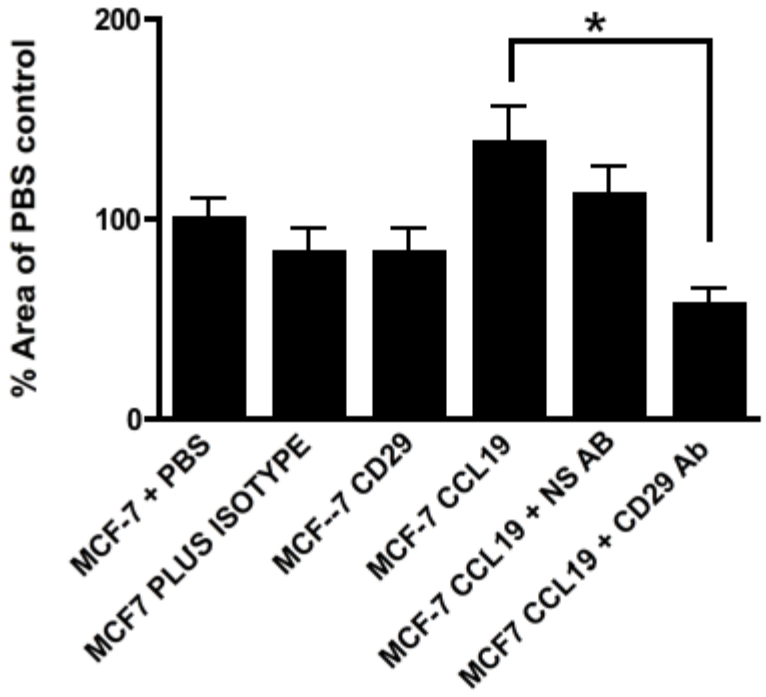

Figure 22. Blocking β1 integrin activation in the presence of CCL19 reduces mammosphere size. Human MCF-7 cells were plated on low density bindging plate in mammosphere media and allowed to grow for 9 days. Mammospheres were fixed and size was measured. Measurement is compared to the percent area of the PBS control.

remained difficult to determine why these women with CCR7 (+) metastases show minimal spread beyond lymphoid organs. From our studies, we find that CCR7 expression leads to increased growth of tumors while promoting migration to the lymph nodes. It is unclear if CCR7 prevents cell death or promotes proliferation. Recently, it has been shown that CCR7 prevents anoikis of MDA-MB-231, MDA-MB-361 and MDA-MB-453 breast cancer cells and blocks apoptosis in hematopoietic cells [166-168]. Since PyVmT-CCR7 mammopsheres grew to significantly larger sizes than vector controls, it will be important to use these mammosphere in the future in biochemical assays to further our understanding of the mechanisms used by CCR7 in breast cancer cells to control the extent of tumor growth.

CCR7 expressing cells travel to lymph nodes and may survive long enough to promote an immunological response, which would correlate with improved survival in human breast cancer patients [154]. It is tempting to speculate that within lymph nodes these CCR7 expressing tumors would co-localize with CCR7-expressing activated dendritic cells, which also express high levels of CCL19. Increased levels of CCL19 have correlated with increased overall survival of human breast cancer patients [169]. It likely that controlling migration of breast cancer cells to CCL19 and CCL21 exposes the tumors to the immune system early during development. It is unclear what events take place within CCR7 (+) breast cancer tumors that control tumor metastases numbers, sites and ability to grow. Due to the inherent nature of CCL19 and CCL21 chemokines, which mediate recruitment of lymphocytes and organogenesis, it is important to consider potential roles for CCR7 in regulation of breast cancer metastasis.

It is unclear at what point CCR7 must be expressed to regulate lymph node metastasis or tumor growth. As mentioned, in our model, PyVmT cells, which lack endogenous CCR7 expression, have been selected that metastasize only to the lung [158]. Therefore, we

hypothesized that expression of CCR7 would lead to increased migration of breast cancer cells to the lymph nodes. It is unclear what function the lymph nodes play in the development of cancer, but we considered several roles. If the lymph nodes were simply filters, where cells were trapped after they exited the tumor, we would have expected to find lymph node metastasis in both PyVmT-CCR7 and PyVmT-vector control mice. In contrast CCR7 could direct metastasis to the lymph nodes, the PyVmT-CCR7 tumors would be able to metastasize to the lymph nodes earlier than the PyVmT-vector cells, and they would provide the immune system with a possible immunogen. In this case they would alert the immune system before the number of PyVmT cells reached a critical level where they induced tolerance in the mouse. Studies are underway in our laboratories to compare the immune response of the mice transplanted with PyVmT-CCR7 to the mice transplanted with PyVmT-vector.

Our results with our mouse model are supported by recent studies in which *in vitro* studies have demonstrated that CCR7 inhibits post intravasation of mammary cancers [170]. In these studies it was also observed that CCR7 failed to affect the growth characteristics of CCR7 expressing tumors in 2D *in vitro* cultures. While they did not examine the metastatic behavior of tumors within the mammary fat pad, in support of our studies, these studies observed that when Her2/neu (CCR7+) cells were injected directly intravenously, the mice had a reduced capacity to develop lung tumors, as well. As mentioned, CCR7 prevents apoptosis but has not been implicated in preventing growth of breast cancer. Therefore, it will be important to determine the mechanisms used by CCR7 (+) tumors that prevent growth in peripheral organs, but allow growth in lymph nodes.

We found that stimulation through CCR7 did not affect adhesion of PyVmT-CCR7 to the β1 integrin, fibronectin in an adhesion assay but that CCR7 promoted migration preferentially to

CCL19 but could mediate migration to CCL21 as well. Since CCR7-expressing tumors grow significantly larger when compared to the vector controls, we asked if exposure of PyVmT-CCR7 to CCL19 or CCL21 could promote proliferation in 3D. To explore the possibility, we examined the growth of PyVmT-CCR7 in mammosphere cultures. We found that similar to what we observed *in vivo*, PyVmT-CCR7 grew to significantly larger mammospheres when compared to vector controls. These results define possible mechanisms used by breast cancer tumors that improve overall survival of breast cancer patients with CCR7 expressing breast cancer. Studies are currently underway to determine to what extent CCR7 controls differentiation of tumors and the signaling events that are activated by CCL19 and CCL21 in breast cancers that mediate the changes in the proliferation rate in 3D cultures.

In conclusion, we have established an MMTV-PyVmT mouse model of breast cancer metastasis that allows us to examine the roles of CCR7 in breast cancer progression and metastasis. We have found that similar to what has been observed in cancer patients, CCR7 can control tumor metastases *in vivo*. In this way CCR7 directs PyVmT-CCR7 cells to the lymph nodes *in vivo*, while CCR7 expressing tumors fail to establish lung metastasis. In order to metastasize, a tumor must change its adhesive properties and detach from the primary. To determine at what step CCR7 controls tumor metastasis we examined the effect of CCR7 stimulation on tumor adhesion.

5.5 Experimental Procedures

5.5.1 Chemicals and Reagents

Chemicals and reagents were purchased from Sigma unless otherwise specified. The following antibodies were used: M2 (anti-FLAG) and fluorescein-conjugated anti-mouse (Jackson Immunoresearch). Murine CCR7 cDNA was a generous gift from James Campbell (Harvard Medical School, Boston, MA). pcDNA3.1 (Invitrogen) or pEGFP-N-1 (Clontech) control vectors, fluorescein- conjugated Donkey anti-mouse (Jackson Immuno Labs) and fibronectin (Sigma) were purchased.

5.5.2 Cell Lines, Mice and *In Vivo* Manipulations

PyVmT cells were generated as described [158]. FVB/N mice were purchased from Jackson labs (Bar Harbor, ME). PyVmT cells were transiently transfected using Lipofectamine *Plus* (Invitrogen) per manufacturer's instructions, with the following modifications. For adhesion assays and *in vivo* injections, cells were plated at 80% density and transfected 16-24 hours later with twice the suggested amount of DNA in serum free DMEM. Cells were incubated in the presence of DNA/Lipofectamine complexes for four hours. The media was adjusted to 10% fetal bovine serum, and the cells were allowed to recover overnight. In the morning, the cells were rinsed with PBS and fresh media was added. Cells were re-transfected each evening on three consecutive days. After the final transfection, cells were trypsinized and plated on Petri dishes overnight at 37C in a 5% CO_2 humidified atmosphere in complete DMEM (10%FBS/90%DMEM/2mM L-glutamine). Cells were isolated with light trypsin, neutralized with cDMEM and injected into the left thoracic mammary fat pad of FVB/N mice (Jackson

Labs). Animals were palpated for tumors starting on day 21, and length, width and height were measured with calipers every other day until the tumors reached 20mm in any measurement. At that point the animals were euthanized, and the tumors and lungs were collected and processed for histology or PCR.

5.5.3 Flow Cytometry

Transfected PyVmT cells were dissociated from tissue culture with cell dissociation solution, rinsed with PBS and labeled with anti-FLAG (M2) antibody (1:1000) for one hour on ice in 1%BSA/PBS. Cells were rinsed 3 x in1% BSA/PBS and labeled with fluorescein-conjugated Donkey anti-mouse (1:200) for one hour on ice 1%BSA/PBS. Cells were rinsed 2 x in 1% BSA/PBS, re-suspended in 200□l 1%BSA/PBS and assayed immediately by flow cytometry (FACS-caliber).

5.5.4 Detection of PyVmT Expression in Lungs and Lymph Nodes

Histological slides were blinded, examined and scored for the presence of metastasis by two individuals. Following physical examination and isolation of the lymph nodes, the DNA was extracted using a Qiagen Tissue isolation kit. DNA was quantified; equal amounts were used to amplify PyVmT DNA as described [158].

5.5.5 Chemotaxis Assays

Transfected PyVmT cells were dissociated from tissue culture with cell dissociation solution, rinsed with PBS and counted. Fifty thousand cells were added in 50µl to the top well of a chemotaxis chamber (Neuroprobe) that had been pre-loaded with a 10µg/ml fibronectin-coated

5µm membrane and increasing concentration of ligand in each well. Cells were allowed to migrate for three hours. At the end of the assay, the media in the top wells was removed, the chamber was disassembled and the cells in the lower wells counted on a hemacytometer.

5.5.6 Mammosphere Cultures

Six million exponentially growing PyVmT cells were isolated by trypsinization, neutralized with complete media, and rinsed twice in PBS and electroporated using a Bio-Rad electroporator according to the manufacturer's protocol (Bio-Rad Bulletin 1349). Transfected cells were isolated by trypsinization and rinsed twice in PBS to remove all traces of serum. Single cells were plated on ultra low-attachment six-well plates (Corning; Corning, NY) at a density of 30,000 viable cells per well. Cells were grown in serum-free mammary epithelial basal medium (Lonza; Walkersville, MD) supplemented with 20 ng/ml EGF, 5 µg/ml insulin, 1 µg/ml hydrocortisone (Lonza), 20 ng/ml bFGF, B27 (Invitrogen; Carlsbad, CA), 4 µg/ml heparin (MP Biomedicals; Irvine CA), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Mediatech). Cells were fed every three days by adding additional media to wells. Seven days after the initial plating, primary mammospheres were measured using a Metamorph. Significance of difference between PyVmT-CCR7 and PyVmT-vector control cells in the presence or absence of ligand was determined using an unpaired *t* test with Welch's correction.

CHAPTER 6 – DISCUSSION AND FUTURE DIRECTIONS

6.1 How the New Conclusions Impact the Field and Future Directions

The function of CCR7 is to mediate lymph node entry and motility within the lymph node, by interacting with the chemokines CCL19 and CCL21. While both ligands are expressed within lymph nodes, CCL21 is also expressed in high endothelial venules, the entry route to lymph nodes. The role of CCL21 in promoting lymph node entry is well established [27, 171]; however, as naive T lymphocytes use CCR7 to mediate migration to CCL21, there is still much to learn about the molecular mechanisms that are initiated upon ligand binding. Dendritic cells express CCL19, which is important for attracting T lymphocytes into close proximity of dendritic cells so that naïve T lymphocytes can become activated [19]. Following dendritic cell/T lymphocyte interactions naïve T lymphocytes become activated, exit from the lymph nodes and migrate to sites of inflammation. Paradoxically, without CCL19, T lymphocytes become trapped in lymph nodes [34, 36], indicating that further information is needed to fully understand the role of CCL19 in lymph node trafficking.

In addition to the role of CCR7 in mediating normal homeostasis of circulating T lymphocytes, CCR7 and its ligands have also been implicated in progression of autoimmune disease. For example, CCR7 knock out mice display decreased severity of antigen induced arthritis (AIA) characterized by decreased knee joint swelling and decreased formation of ectopic tertiary lymphoid structures [172]. In support of these findings, CCL21 has been shown to promote lymphoid neogenesis in pancreatic islets, which serves to recruit other inflammatory

mediators to the pancreas and perpetuates the autoimmune state [35]. In addition, CCL21 expression is increased on lymphatic endothelial cells in Myasthenia gravis and is thought to promote thymic hyperplasia [173]. Similarly, both CCR7 and CCL21 expression are increased in graft versus host disease and atopic dermatitis, also serving to recruit other inflammatory mediators such as central memory T cells, which maintains the inflammatory response [146]. In contrast, studies using CCR7 knock out mice revealed that in the absence of CCR7 mice have greater immune infiltrates in the stomach, lung, pancreatic islets and liver, which is a hallmark of autoimmunity [174]. Additionally, CCR7 knock out mice spontaneously developed a chronic renal autoimmune disorder, similar to systemic lupus erythematosus characterized by glomerular IgG deposits, reduction in creatinine clearance and anti-nuclear antibodies [174]. Because the presence of CCR7 in some instances seems to yield a protective function against autoimmunity, yet in other cases the presence of CCR7 increases the severity of the disease, CCR7 and its ligands represent important targets for investigation.

Similar to T lymphocytes, metastatic breast cancer cells also express CCR7. It remains unclear if CCR7 expression by breast cancer cells promotes metastasis to lymph nodes *in vivo* and what affect this has on disease outcome. Therefore, using what we learn about CCR7 mediated migration in T lymphocytes can be used to assist in understanding how breast cancer cells also use CCR7 to mediate migration to CCL19 and CCL21 and perhaps how to control metastasis.

The ultimate goal of the work outlined in this dissertation is to contribute to a greater understanding of CCR7 mediated migration outlined in the following questions: 1) What are the mechanisms by which naïve T lymphocytes are attracted to migrate into lymph nodes? 2) Does CCR7 contribute to egress from the lymph node? 3) In breast cancer is CCR7 required for

lymph node metastasis and does this promote or prevent metastasis to other organs? 4) Do metastatic breast cancer cells hijack CCR7 mechanisms used by T lymphocytes to mediate metastasis to lymph nodes? The results of my dissertation contribute toward the resolution of some of these questions.

6.1.1 What are the Mechanisms by Which Naïve T Lymphocytes are Attracted to Migrate into Lymph Nodes?

Naive T lymphocytes enter into lymph nodes through high endothelial venules, which express CCL21. Additionally, high endothelial venules also express fibronectin [9], which is the ligand for the β1 integrin. Integrins are important adhesion proteins activated by chemokine receptors during migration. As cells migrate they use integrins to adhere to the extracellular matrix, which includes proteins such as fibronectin [175]. The cell then propels forward, de-attaches from the matrix by de-activating the integrins [6]. Then the integrins recycle back into the cell in preparation for the next migration cycle [176]. Because high endothelial venules express both CCL21 and fibronectin, it is important to understand how and if naïve T lymphocytes use CCL21 and fibronectin ultimately to gain entry into lymph nodes.

While several studies have revealed downstream signaling effectors of CCR7 activation, there has been a lack of understanding if these signaling molecules are important for naïve T lymphocyte migration. For example, ERK1/2 has been shown to be transiently phosphorylated in response to CCR7 stimulation by CCL21 in primary T lymphocytes and in HEK293 cells [49, 54], however it is unclear from these studies if phosphorylation of ERK1/2 is necessary for

migration. Similarly, PLCγ1 has been shown to be activated by CCR7 in response to CCL21 in highly motile cancer cells [83], yet a possible role in CCR7 mediated T lymphocyte migration has not been defined. The possible role of PLCγ1 in migration is suggested by its ability to bind to and activate β1 integrins [62]. Additionally, T lymphocyte activation of CCR7 by CCL21 leads to adhesion and spreading on VCAM-1, a β1 integrin ligand [4].

We have found that migration to CCL21 on fibronectin requires ERK1/2, PLCγ1 and Gαi activation, however only PLCγ1 is required for β1 integrin activation (Figure 23). We observed that rapid and transient phosphorylation of ERK1/2 is required for migration of naïve T lymphocytes to CCL21 on fibronectin, but is not required for initial activation of β1 integrins. In addition, we found that inhibition of PLCγ1 resulted in a loss of naïve T lymphocyte migration to CCL21 and β1 integrin activation. Inhibition of PLCγ1 also resulted in sustained phosphorylation of ERK1/2 confirming the importance of transient phosphorylation of ERK1/2 for migration and suggesting an important role for the recruitment of a phosphatase in response to PLCγ1 activation. In the future it will be important to identify the phosphatase responsible for de-phosphorylating ERK1/2. Two possible phosphatase candidates that could be regulated by PLCγ1 include dual specificity phosphatase 2 (PAC-1) and mitogen-activated protein kinase phosphatase (MKP-3), which are expressed in T lymphocytes and specifically target ERK1/2 [177].

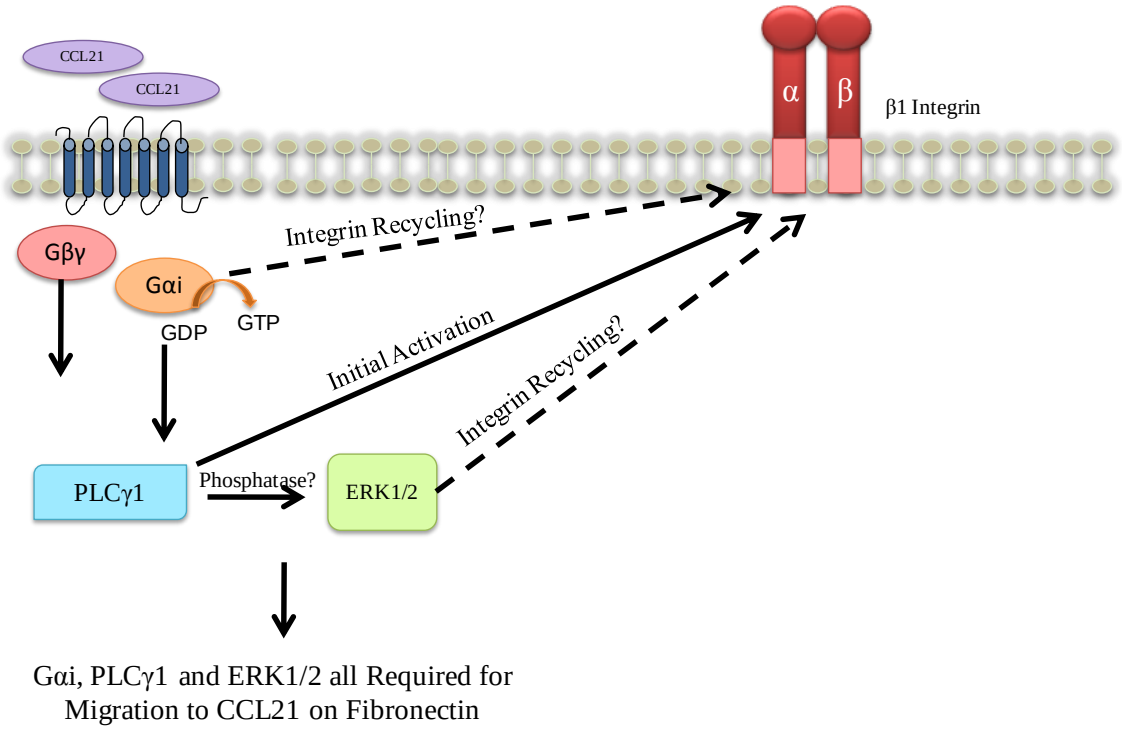

Figure 23. Proposed CCR7/CCL21 signaling in naïve T lymphocytes. We propose that activation of CCR7 by CCL21 results in Gαi activation , which results in down-stream phosphorylation of PLCγ1, which in turn results in down-stream transient phosphorylation of ERK1/2. Inhibition of Gαi, PLCγ1 or ERK1/2 results in a loss of migration to CCL21 on fibronectin. Only inhibition of PLCγ1, but not Gαi or ERK1/2 inhibition, prevents β1 integrin activation. We propose that inhibition of Gαi or ERK1/2 may prevent recycling of β1 integrins, thus preventing migration to CCL21 on fibronectin.

Although CCR7 is well established to mediate migration of T lymphocytes via Gαi coupling to the receptor, the downstream signaling events resulting from Gαi activation that are required for migration to CCL21 have remained unclear. We found that both PLCγ1 and ERK1/2 phosphorylation was reduced, but not completely lost upon inhibition of Gαi in response to stimulation with CCL21. This suggests that another pathway may also contribute to activation of PLCγ1 and ERK1/2. Other signaling pathways that activate PLCγ1 in T lymphocytes includes stimulation of growth factor receptors and T cell receptors [178]. Because inhibition of Gαi reduced migration to CCL21 without preventing the initial activation of β1 integrins, one of these other signaling pathways may have initiated β1 integrin activation while Gαi-induced PLCγ1 activation. Other Gαi-dependent signaling pathways may be important for de-attaching or recycling of β1 integrins.

Another possible mechanism for Gαi-independent activation of β1 integrins is activation of the Janus Kinase-2 (JAK-2) non-receptor tyrosine kinase [179], which could also explain the reduction, but not complete loss of PLCγ1 and ERK1/2 phosphorylation in response to pertussis toxin. Several studies report that ligand binding to GPCRs initiate the traditional G-protein pathway and the JAK/STAT signaling pathway [180-182]. Interestingly, inhibition of JAK-2 kinase resulted in decreased β1 integrin activation independent of Gαi activation in response to CCL21 stimulation of CCR7 [179], providing a possible explanation for our results. In the future it will be important to carry out studies to inhibit both Gαi and JAK-2 in order to understand if these pathways work together to contribute to β1 integrin mediated migration to CCL21 on fibronectin. Additionally, it will be important to further understand how inhibition of JAK-2 affects PLCγ1 and ERK1/2 signaling.

Integrins are unique receptors in that they undergo bi-directional signaling. Integrins can be signaled to from another receptor, such as CCR7 a process termed "inside out" signaling. Integrins can also signal through "outside in" signaling via interaction with a ligand. Our work demonstrates that CCL21 signals to activate β integrins through "inside out" signaling. However β1 integrins also signal upon contact with fibronectin which also contributes to CCR7 motility through "outside in" signaling. It will be important to further understand how these two pathways converge to mediate migration by co-incubating both CCL21 and fibronectin and observe changes in signaling compared to fibronectin or CCL21 alone. β1 integrins also phosphorylate PLCγ1 in order to mediate motility [84], so one could speculate that *in vivo* when a T lymphocyte encounters CCL21 and fibronectin both signals combined further amplify activation of PLCγ1 in order to promote continuous migration. This is significant, because as naïve T lymphocytes migrate through the high endothelial venules to enter into lymph nodes, the lymphocytes will encounter both CCL21 and fibronectin signals at the same time and in the future it may be important to not only inhibit CCL21, but also inhibit β1 binding to fibronectin to completely inhibit migration.

Taken together, our working model is that PLCγ1 is required for migration to CCL21 and activation of β1 integrins, while Gαi and ERK1/2 are required for migration. PLCγ1 promotes migration by terminating phosphorylation of ERK1/2 via activation of an unknown phosphatase. Gαi contributes to migration by activating PLCγ1. At this point it remains unclear as to the mechanism of how ERK1/2 is phosphorylated since Gαi inhibition only partially decreases ERK1/2 activation. Further, it is uncertain if ERK1/2 is continually cycling between phosphorylated and de-phosphorylated states or if once de-phosphorylated, ERK1/2 remains de-phosphorylated for the duration of the migration event. It is also unclear if PLCγ1 directly

activates the phosphatase that de-phosphorylates ERK1/2 or if there is a signaling intermediate. It will be important to identify other signaling molecules activated by CCR7 in response to CCL21 in order to better understand how PLCγ and ERK1/2 contribute to migration.

This work contributes to the greater understanding of the mechanisms required for T lymphocyte migration and in the future it will be important to continue to define mechanisms that are important for migration to CCL21. As CCL21 is important for T lymphocyte entry into lymph nodes, it is important to understand if blocking this event could alter the course of an immune response. An antagonist of CCL21 (mSLC4) was found to reduce the severity of graft versus host disease in mice, which was contributed to a reduction in CD4+ cells, which in turn reduced activated B lymphocytes [37]. Perhaps one could develop specific targets against the T cell receptor that recognizes a specific self antigen, coupled with a CCL21 antagonist. This would allow one to specifically target those T lymphocytes engaged in attacking the body and prevent them from entering into lymph nodes to become activated without affecting the entire immune system. Therefore, CCL21 represents an important target for manipulation of the immune response. Certainly, more work is needed to continue to increase our knowledge of CCL21 mediated mechanisms that will hopefully serve to identify specific inhibitors that can possibly be used to treat disease in the future.

6.1.2 Does CCR7 Contribute to Egress From the Lymph Node?

As mentioned during normal homeostasis naïve T lymphocytes are in continuous migration throughout the lymph nodes. Although it is very important that these cells are able to

migrate into lymph nodes, it is imperative they also be able to migrate out in order to continue to scan for antigen and carry out effector functions in the periphery. During an immune response naïve T lymphocytes enter into lymph nodes and immediately begin scanning dendritic cells that present antigen. The close proximity of dendritic cells to the high endothelial venules, the route by naïve T lymphocytes enter into lymph nodes, as well as expression of CCL19 by dendritic cells facilitate these interactions [19, 183]. Once T lymphocytes recognize antigen they form stable interactions with dendritic cells that can last upwards of 36-48 hours [22]. During this prolonged interaction the T lymphocyte becomes activated, begins to proliferate and eventually detaches from the dendritic cell [23]. Lastly, the activated T lymphocytes exit the lymph nodes and return to the circulation to carry out their effector functions in the periphery.

The $S1P_1$ receptor has been well defined for its role in promoting lymph node egress [65, 123]. When naïve T lymphocytes first enter into the lymph nodes, $S1P_1$ levels are low so as to not cause exit prematurely before having the opportunity to locate antigen [184]. However, after some point when the cell receives a signal to exit, $S1P_1$ is up-regulated. The molecular mechanisms that mediate up-regulation of $S1P_1$ in order to initiate lymph node egress are still not understood. Two separate studies carried out with a CCL19-deficient mouse and a CCL19 antagonist (8-83) demonstrated that without CCL19 function T lymphocytes were retained in lymph nodes during normal homeostasis and during immune responses [34, 36]. Furthermore, in CCR7-deficient mice, $S1P_1$ levels were also found to be decreased [69], suggesting that CCR7 expression may be important for $S1P_1$ expression.

Together, these studies suggest a role for CCR7/CCL19 in mediating lymph node egress. We first made the observation that T lymphocytes treated with CCL19, but not CCL21 migrate to S1P, at 48 hours, suggesting that CCL19-treated cells had increased expression of $S1P_1$.

Importantly, CCL19-treated cells did not migrate to S1P until 48 hours after treatment. The significance of this timing correlates with T lymphocytes losing the ability to migrate to S1P *in vivo* 24 hours after entering into lymph nodes [64], then regaining the ability to migrate to S1P by 72 hours [65]. To investigate the mechanism of up-regulation of $S1P_1$ we assayed for changes in protein expression of ERK5 and KLF-2 following treatment with CCL19. We found that activation of CCR7 by CCL19 over the course of 24-72 hours increased phosphorylation of ERK5 and up-regulated total levels of both ERK5 and KLF2, suggesting a mechanism for the increase in $S1P_1$ levels and increased migration observed to S1P at 48 hours (Figure 24). Our proposed ERK5 pathway was further supported when we used siRNA to knock down ERK5 and found that CD4+/CD8+ T lymphocytes failed to exit lymph nodes and displayed decreased migration to S1P 48 hours following CCL19 treatment.

Taken together, our results suggest that CCR7activation by CCL19 is important for turning on the machinery that regulates lymph node exit via $S1P_1$. In the future, it will be important to determine the impact of inhibiting the CCR7/CCL19/ERK5 pathway on the immune response or other cellular functions. This is of great interest since FTY720, an inhibitor of $S1P_1$, is currently being used in phase III clinical trials to treat multiple sclerosis [185, 186]. It is interesting to speculate which ligand, CCL21 or CCL19 or both, would be most effective at inhibiting the immune response. As CCL21 is important for entering the lymph nodes one could argue that preventing these cells from ever entering the lymph nodes would be important for diminishing any possible immune response. However it seems that during immune responses T lymphocytes can find alternative ways to enter into lymph nodes such as through the afferent lymphatics and can bypass CCR7 ligands altogether [187], which might still result in enough T lymphocyte activation to cause damage.

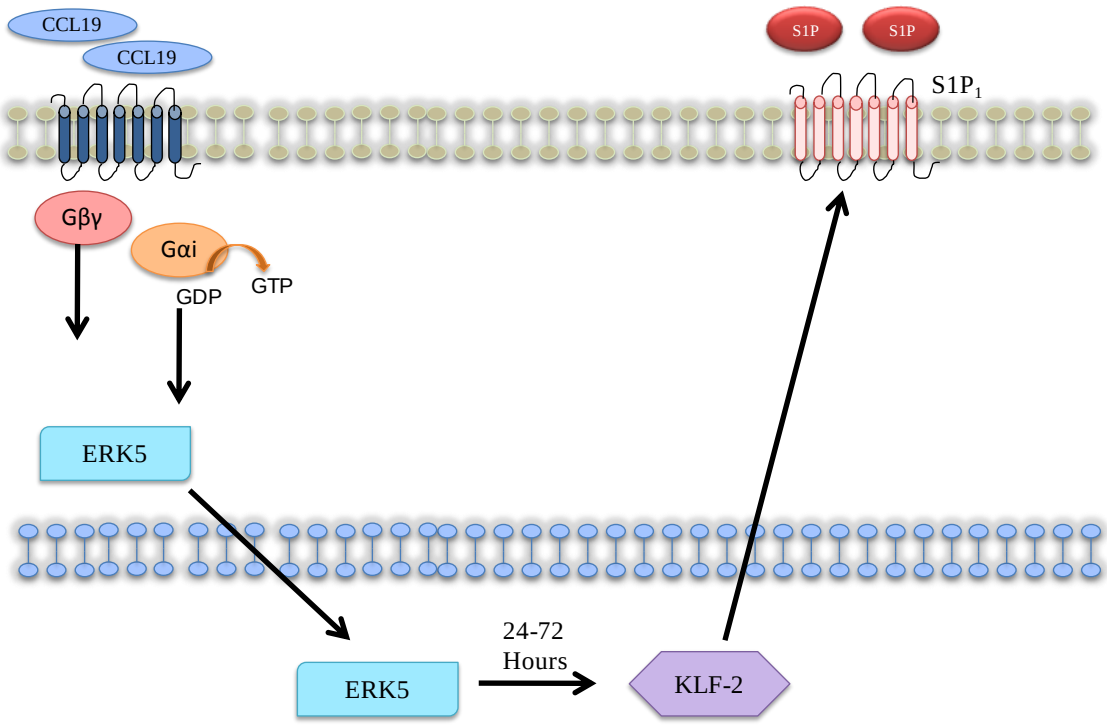

Figure 24. Proposed CCL19/CCR7 signaling required for increased expression of $S1P_1$. We propose that activation of CCR7 by CCL19 results in phosphorylation of ERK5, which increases transcription of KLF-2, which in turn promotes increased transcription of $S1P_1$. We found that treatment of Hut78 human T lymphocytes and primary murine T lymphocytes with CCL19 over the course of 48 hours increased phosphorylation of ERK5 and increased overall total protein levels of ERK5. We observed also that KLF-2 protein levels were increased over this same time period. After 48 hours of CCL19 treatment, cells were able to migrate to S1P, suggesting increased $S1P_1$ receptor on the surface of these cells. Increased $S1P_1$ mRNA was confirmed by RT-PCR. Knock down of ERK5 resulted in decreased migration of T lymphocytes to S1P at 48 hours and retention of CD4+ and CD8+ T lymphocytes in lymph nodes.

Therefore, one might argue that inhibiting both ligands would provide the best alternative to not only prevent entry but for those lymphocytes that do enter, also block exit to ensure that you could control the entire T lymphocyte response. One important consequence of inhibiting CCL21 and CCL19 together is that in *plt* mice, which lack both ligands CCL19 and CCL21, immune responses are delayed initially but ultimately enhanced over a period of 20 days [187], which would be detrimental in autoimmune disease. As the *plt* mouse is a result of a spontaneous mutant that deletes both CCL19 and CCL21, it is unclear if both ligands contribute to the delayed but enhanced response. Therefore, it will be important in the future to use the antagonists CCL19 (8-83) and CCL21 (mSLC4) together and individually to understand if the impact of blocking both ligands or simply one ligand CCL19 or CCL21 results in a similar enhanced response. In contrast, a delayed but enhanced immune response would be very beneficial with respect to vaccinations and even possibly to boost immunity during bacterial and viral infections. Taken together, there are still many questions that need to be answered, but our findings contribute to the current knowledge of how T lymphocytes emigrate into the periphery and define additional potential therapeutic targets that could possibly be used in either in combination with FTY720 or alone to treat autoimmune disease.

6.1.3 In Breast Cancer is CCR7 Required for Lymph Node Metastasis and Does This Promote or Prevent Metastasis to Other Organs?

Recent studies have found strong correlations between patients who develop CCR7 expressing breast cancers and metastasis to lymph nodes, which highly express CCR7 ligands. [73, 75]. However, a contrasting study determined that CCR7 expression did not correlate with

lymph node metastasis in breast cancer [76]. At present, it is unclear if and by what mechanisms CCR7 controls lymph node metastasis in breast cancer. We have demonstrated in a mouse model that CCR7(+) breast cancer cells preferentially metastasize to lymph nodes, while CCR7(-) cells metastasized to the lung and were not found in the lymph node. Several studies have reported that CCR7(+) tumors result in overall decreased patient survival however, it remains uncertain as to why patient survival is decreased [73]. In contrast, other studies suggest that CCR7(+) tumors have no effect on overall survival [154]. In most breast cancer cases, axillary lymph nodes are removed without any understanding if removal affects overall patient survival [74]. Unexpectedly, we found that while CCR7(+) mice that had preferential metastases to the lymph nodes, that lung metastases were reduced.

As lymph nodes are important sites for activation of lymphocytes, it seems counter intuitive that cancer cells would dare up-regulate receptors that would allow migration into these areas, as these are places of highly activated lymphocyte, that could potentially kill the cancer cells right then and there. Therefore, it is possible that in our model CCR7(+) cells were directed into the lymph nodes where they come into direct contact with lymphocytes that resulted in the mounting of a stronger immune response against the metastatic cancer cells, thus preventing further metastases to distant organs.

Alternatively, one could argue that cancer cells migrating into lymph nodes could provide an advantage for the cancer cells, such as inducing immunologic tolerance. Tolerance is characterized by the lack of an immune response directed toward specific antigens, such as tumor antigen. The induction of tolerance may provide an explanation as to why in some cases CCR7(+) tumors correlate with a worse patient outcome. It is difficult at this stage to fully understand the dynamics of what is occurring in lymph nodes when breast cancer metastases

migrate to these locations. It will be important to analyze the immune response in CCR7(+) breast cancers to understand if the immune response is enhanced or down-regulated as compared to CCR7(-) breast cancers. For example, one could begin by using the $CCR7^{-/-}$ mouse model and assaying for numbers of T regulatory cells, which are important for down-regulating the immune response. Additionally, one could isolate CD8+ cytotoxic T cells from mice who have developed tumors and perform cytotoxic killing assays with PyVmT cells isolated from CCR7+ and CCR7- tumors and metastasis. Furthermore, because we found that lymph nodes metastases resulted in decreased metastases to the lung, more work is needed to fully understand the impact of removal of lymph nodes during disease progression, as we are not yet certain if this affects the immune response and/or metastatic outcome CCR7(+) breast cancer patients. Removal of lymph nodes could result in increased metastases to more distant locations simply because cancer cells that would normally migrate into lymph nodes are possibly directly exposed to antigen presenting cells that could take up antigen from dead or dying cancer cells and effector cytotoxic cells that could directly kill the cancer cell. Ultimately much more work is needed to fully understand the role of CCR7 and the role of lymph nodes in breast cancer metastasis.

6.1.4 Do Metastatic Breast Cancer Cells Hijack CCR7 Mechanisms Used by T Lymphocytes to Mediate Metastasis to Lymph Nodes?

As mentioned in Chapter 1, lymphocyte migration is a complex process in which the cell activates adhesive contacts to adhere to extracellular molecules [1]. The cell then spreads in order to be propelled over the extracellular matrix and then de-activates its adhesive contacts to release from the extracellular matrix, to repeat the cycle again [7]. In naïve T lymphocytes, both

CCR7 and β1 integrins are important for mediating migration into lymph nodes. Naïve T Lymphocytes enter into lymph nodes via high endothelial venules, which express CCL21 as well as fibronectin, a β1 integrin ligand [9]. Importantly, CCR7 has been shown to mediate migration of T lymphocytes to CCL21 on a β1 integrin ligand. Specifically, T lymphocyte activation of CCR7 by CCL21 leads to adhesion and spreading on VCAM-1, a β1 integrin ligand, resulting in migration to CCL21 [4]. In response to CCR7 activation by CCL21, T lymphocytes activate several important mediators that regulate migration and β1 integrin activation. For example, we observed that both ERK1/2 and PLCγ1 are necessary for migration to CCL21 on the β1 integrin ligand fibronectin. In addition, JAK-2 is required for β1 integrin activation in response to CCL21 activation and is required for migration to both CCL19 and CCL21 [179].

Similar to T lymphocytes, metastatic breast cancers also express CCR7 and β1 integrins. In contrast to the primary tumor, metastatic breast cancer cells display increased expression of β1 integrins on their cells surface [85]. Blocking β1 integrins reverts the malignant phenotype of these breast cancer cells to a normal epithelial phenotype, characterized by well organized actin, organized adherens junctions, deposition of a basement membrane and growth arrest [85]. Therefore, it is important to understand how β1 integrins are regulated in breast cancer cells and use our knowledge of how T lymphocytes signal to and regulate β1 integrins so that we can begin to understand this process in breast cancer.

While breast cancer cells migrate to CCL19 and CCL21, it is not clear if breast cancer cells can signal through CCR7 to regulate β1 integrin mediated adhesion or migration similar to T lymphocytes. Migration is a multi-step process in which cells must adhere, spread and de-adhere with each step relying greatly on integrin interactions with extracellular matrix molecules. We found that activation of CCR7 was not important for β1 mediated cell adhesion or initial

activation of the β1 integrin in PyVmT murine breast cancer cells. However, CCR7 activation by CCL19 did promote β1 integrin-specific spreading. These results suggested that CCR7 signals to β1 integrins to promote migration. In support of this model, we found that CCR7-induced breast cancer cell migration to both CCL19 and CCL21 on fibronectin. *In vivo* T lymphocytes travel into lymph nodes via high endothelial venules which express CCL21 and fibronectin. Therefore, these results support our mouse model results from the previous section in which CCR7 promoted lymph nodes metastasis. Furthermore, these results suggest that CCR7 can promote migration, similar to that seen with T lymphocytes, under similar conditions presented in high endothelial venules. In the future it will be important to begin to understand the molecular mechanisms that contribute to CCR7-mediated migration to both CCL19 and CCL21. For example, it will be important to understand the role of PLCγ1, as it is not only important during CCR7/CCL21 activation and mediated migration, but is also activated in response to β1 integrin binding of ligands. Additionally, ERK1/2 is also activated in response to CCL19 and CCL21 stimulation of CCR7 in T lymphocytes and may play an important role in breast cancer migration as well.

One hallmark of cancer is the ability to undergo uncontrolled cell proliferation. In metastatic breast cancer cells β1 integrins promote proliferation [188]. Since we observed CCR7 mediated migration on β1 integrins, suggesting the possibility that CCR7 signals to β1 integrins, we questioned if activation of CCR7 by CCL19 or CCL21 could promote growth of breast cancer cells, possibly via a β1 integrin mediated mechanism. We found that both CCL19 and CCL21 promoted growth of breast cancer cells, while β1 integrin inhibition decreased growth in the presence of CCL19. These results were further supported *in vivo* as CCR7(+) breast cancer tumors displayed an increase in tumor volume compared to CCR7(-) tumors. It is important to

take into consideration that increased growth could be a result of increased proliferation, increased survival or decreased death signals. Therefore, it will be important to assay for changes in anti-apoptotic mediators such as Bcl-2, apoptotic mediators such as caspase-3 and proliferation markers such as Ki-67 in order to further understand how activation of CCR7 can affect growth in breast cancer cells.

Taken together, our data suggest that CCR7 mediates spreading, migration and growth of metastatic breast cancer cells. In the future it will be important to continue to learn what CCR7-mediated mechanisms are important for breast cancer migration and metastasis as this knowledge could be used to develop drug targets. This will help us to understand to a greater extent if CCR7 behavior in breast cancer cells will resemble that observed in T lymphocytes. This is significant, because if CCR7 becomes a therapeutic target for metastatic breast cancer and CCR7 activates similar signaling pathways in T lymphocytes, the immune system will also be affected with any drugs developed to target CCR7 expressing breast cancer cells.

6.2 Concluding Remarks

Taken together, the work carried out in this dissertation contributes to a deeper understanding of the molecular mechanisms that are carried out in response to activation of CCR7 by its ligands CCL19 and CCL21. Furthermore, we have identified a novel and important function of the CCR7 receptor in naïve T lymphocytes, to regulate lymph node egress. We have also learned that CCR7 promotes breast cancer metastasis to lymph nodes, while reducing metastasis to other vital organs. If metastatic breast cancers use similar CCR7 mediated

mechanisms as T lymphocytes to mediate egress from lymph nodes, the findings outlined in this dissertation could eventually lead to additional molecular targets for cancer therapies. Currently, a S1P specific antibody (ASONEP) has been found *in vivo* to reduce metastasis and is currently in phase 1 clinical trials [189]. Overall the information presented in this dissertation provides new insights, yet also raises important questions that hopefully someday will lead to a better resolution for autoimmune disorders and cancer metastasis.

CHAPTER 7 – AUTHOR CONTRIBUTIONS

7.1 CCR7/CCL21 Migration on Fibronectin is Mediated by PLCγ1 and ERK1/2 in Primary T Lymphocytes

Authors: Laura A. Shannon[(1)] Psachal A. Calloway[(1)] and Charlotte M. Vines[(1)]

LAS Completed:

Carried out all migration assays (UO126, Pertussis, U73122 and PLCγ1 shRNA) in Hut78 and primary cells, all signaling studies +/- inhibitors and shRNA in primary cells , all β integrin activation studies +/- inhibitors and shRNA in primary cells, study design, data analysis, preparation and revisions of the manuscript.

PAC Completed:

Initial studies in the Hut78 Cell line to show that PLCγ1 was phosphorylated by CCL19/CCL21.

CMV Completed:

Carried out study design, data analysis, manuscript revisions and submission of the manuscript

7.2 CCR7/CCL19 Mediates T Lymphocyte Expression of EDG-1

Authors: Laura A. Shannon, Melissa A. Byers, Brian C. Fassold, Megan D. Roth, Psachal A. Calloway, Antoine Perchellet and Charlotte M. Vines

LAS Completed:

Carried out RT-PCR, retroviral amplification and characterization, signaling assays (activation of ERK5 and KLF-2 in primary cells), designed and carried out the T cell/dendritic cell conjugation assays, migration assays to S1P, designed and carried out adoptive transfer assays, involved in data analysis, study design and manuscript preparation.

MAB Completed:

Designed T cell/dendritic cell assays.

BCF completed:

Carried out all Hut78 signaling assays.

MDR completed:

Carried out Hut78 KLF2 signaling assays.

PAC Completed:

Carried out Hut78 ERK1/2 signaling assays.

AP Completed:

Carried out adoptive transfer assays.

CMV Completed:

Carried out signaling assays, migration assays, designed and carried out adoptive transfer assays, FTOC assays, data analysis, study design, manuscript preparation and submission.

7.3 Expression of a Chemokine Receptor, CCR7, Mediates Metastasis of Breast Cancer to the Lymph Nodes and Reduces Metastasis to the Lungs in mice

Authors: Heather D. Cunningham, M.D.[1,3*], Laura A. Shannon, B.S.[2,3*], Tiffany McBurney, M.S. [2], Psachal A. Calloway B.S.[2], Brian C. Fassold[2], Irene Dunwiddie,M.S.[4], George Vielhauer Ph.D.[4], Ming Zhang Ph.D.[5], Charlotte M. Vines Ph.D.[2]

*HDC and LAS are co-first authors

HDC Completed:

Carried out transient transfection of PyVmT cells, adhesion and spreading assays (PyVmT), mammosphere assays, histological analysis, development of animal model, manuscript and study design.

LAS Completed:

Carried out transient transfection of PyVmT cells, PyVmT 2D proliferation studies, migration assays (PyVmT, MCF-7 and MCF-10A) +/- fibronectin, mammosphere assays (PyVmT, MCF-7, MCF-10A and MDAMB231) +/- β1 integrin blocking antibody, designed and carried out FVB mouse injection model, animal surgery, measured tumors, harvested organs, manuscript and study design.

PAC Completed:

Carried out transient transfection of PyVmT cells and measured tumors.

BCF Completed:

Measured tumors.

ID and GV Completed:

Injected PyVmT cells into FVB mice.

MZ Completed:

Developed PyVmT/FVB mouse model of lung metastasis.

CMV Completed:

Carried out migration assays, mammosphere assays, designed and carried out FVB mouse injection model, animal surgery, measured tumors, harvested organs, transient

transfection of PyVmT cells, 2D proliferation studies, study design and manuscript preparation and submission.

CHAPTER 8 - LITERATURE CITED

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