THE ROLE OF PSGL-1 IN T CELL MIGRATION AND HOMEOSTASIS

by

KRYSTLE VEERMAN

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ABSTRACT

P-Selectin Glycoprotein Ligand-1 (PSGL-1) is an extracellular glycoprotein expressed on most leukocytes that is important for rolling and tethering of activated leukocytes to areas of inflammation through direct interactions with P-selectin, E-selectin and L-selectin. However, PSGL-1\textsuperscript{null} mice showed subtle defects in their homeostatic T cell profile that cannot be adequately explained by a defect in migration of activated leukocytes. PSGL-1\textsuperscript{null} mice had a reduced number of CD4+ and CD8+ T cells in the peripheral blood but not in the lymph nodes and spleen. T cell subset analysis revealed that there was a severe reduction of naïve T cells in the peripheral blood and a moderate reduction of naïve T cells in the lymph nodes whereas other T cell compartments were not as affected.

Here we determined that PSGL-1 was important for the homing of T cells to secondary lymphoid organs and that PSGL-1 functioned independently of selectin interaction. PSGL-1 enhanced T cell homing through an interaction with secondary lymphoid chemokines CCL21 and CCL19. We have also shown that PSGL-1\textsuperscript{null} T cells are delayed in leaving the lymph nodes suggesting PSGL-1 is important in the movement of T cells both in and out of lymph nodes. Therefore, the reduced ability of PSGL-1\textsuperscript{null} T cells to enter lymph nodes and receive survival factors may contribute to disturbed T cell subsets in peripheral blood and lymph nodes of PSGL-1\textsuperscript{null} mice.

There is evidence of spontaneous CD8+ T cell specific proliferation in PSGL-1\textsuperscript{null} cells in lymphoreplete environments. This appears to be caused by a combination of factors, namely an increase in lymph node residence time as well as a more rapid proliferative response to the homeostatic cytokines IL-15, IL-2 and IL-4. However, PSGL-1\textsuperscript{null} T cells are also less viable both
in vivo and in vitro. Therefore, spontaneous proliferation may be a compensatory mechanism that balances T cell levels to normal in lymph nodes and spleens of PSGL-1\textsuperscript{null} mice.

These findings collectively demonstrate a novel, selectin-independent role for PSGL-1 on T cells under non-inflammatory conditions both in T cell homing to secondary lymphoid organs as well as maintenance of T cell homeostasis.
PREFACE

Chapter 3 includes data published in: Veerman, K. M., M. J. Williams, K. Uchimura, M. S. Singer, J. S. Merzaban, S. Naus, D. A. Carlow, P. Owen, J. Rivera-Nieves, S. D. Rosen, and H. J. Ziltener. 2007. Interaction of the selectin ligand PSGL-1 with chemokines CCL21 and CCL19 facilitates efficient homing of T cells to secondary lymphoid organs. *Nat Immunol* 8:532-539. J.S.M., H.J.Z. and K.U., S.D.R. independently discovered the PSGL-1 requirement for T cell homing; K.M.V. did the competitive homing assays; K.U. and M.S.S. did the mAb inhibition homing assays; M.J.W. designed and did the chemotaxis and CCL21 binding assays; S.N. contributed to the CCL21 binding studies; D.A.C. contributed to the experimental design of the competitive *in vivo* and *in vitro* studies; P.O. produced all chemokines; J.R.-N. provided anti-PSGL-1 and helped with the inhibition studies; H.J.Z. and S.D.R. supervised research and coordinated ongoing work; K.M.V. and H.J.Z. wrote the first draft of the manuscript; and all authors contributed to discussions and preparation of the manuscript.

Figures 3.6A&C; 3.8A,B&D; 3.9A, B, C; 3.10A&B; 3.11; 3.12 have been published in the aforementioned manuscript.

Figures 3.9A, B, C; 3.10A&B; 3.11; 3.12 were produced by Michael Williams in our laboratory.

Chapter 1 includes work published in: Carlow, D. A., K. Gossens, S. Naus, K. M. Veerman, W. Seo, and H. J. Ziltener. 2009. PSGL-1 function in immunity and steady state homeostasis. *Immunol Rev* 230:75-96. All authors contributed literary reviews to the manuscript. K.M.V. contributed sections on PSGL-1 signaling and critical review of the manuscript.

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<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C2GnT</td>
<td>Core 2 β1,6-N-acetylglucosaminylsugartransferase</td>
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<td>CCL19</td>
<td>Chemokine (C-C motif) ligand 19 (CCL19)</td>
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<td>CCL21</td>
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<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CTO</td>
<td>cell tracker orange</td>
</tr>
<tr>
<td>CTP</td>
<td>common T cell progenitor</td>
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<td>Chemokine (C-X-C motif) ligand 12</td>
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<td>DN</td>
<td>CD4 ,CD8 double negative</td>
</tr>
<tr>
<td>DOP</td>
<td>4’ deoxypuridoxine</td>
</tr>
<tr>
<td>DP</td>
<td>CD4 ,CD8 double positive</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetra acetic acid</td>
</tr>
<tr>
<td>ELC</td>
<td>Ebstein-Barr virus-induced molecule 1 ligand chemokine</td>
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<tr>
<td>ELP</td>
<td>early lymphoid progenitor</td>
</tr>
<tr>
<td>ELR motif</td>
<td>glutamic acid-leucine-arginine motif</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>ezrin, radixin, and moesin</td>
</tr>
<tr>
<td>ESL-1</td>
<td>E-selectin ligand-1</td>
</tr>
<tr>
<td>ETP</td>
<td>early T lineage progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fli3</td>
<td>fms-like tyrosine kinase-3</td>
</tr>
<tr>
<td>FRC</td>
<td>fibroblastic reticular cell</td>
</tr>
<tr>
<td>FucT</td>
<td>fucosyltransferase</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GlyCAM-1</td>
<td>glycosylation-dependent cell adhesion molecule-1</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoproteins</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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HEV  high endothelial venule
HIV  human immunodeficiency virus
HSC  hematopoietic stem cell
ICAM  intercellular adhesion molecule
IFN  interferon
IL  interleukin
ITAM  immunoreceptor tyrosine-like activation motif
JAK  janus kinase
LCMV  lymphocytic choriomeningitis virus
LDL  low density lipoproteins
LFA-1  lymphocyte function-associated antigen 1
LIP  lymphopenia induced proliferation
LN  lymph nodes
LPS  lipopolysaccharides
LSK  Lin- Sca-1+ c-Kit+
mAb  monoclonal antibody
MA
dCAM-1  mucosal addressin cell adhesion molecule 1
MCL-1  myeloid leukemia cell differentiation protein
MHC  major histocompatibility complex
MIP-3-β  macrophage inflammatory protein-3-β
MPP  multipotent progenitor
Naf-1  Nef-associated factor 1
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
NK  natural killer
PALS  periarteriolar lymphoid sheath
PBS  phosphate buffered saline
PI  propidium iodide
PI3K  phosphatidylinositol 3-kinase
plt  paucity of lymph node T cells
PNAd  peripheral node addressin
PP  Peyer’s patches
PSGL-1  P-selectin glycoprotein ligand-1
Rag  recombination activating gene
RPMI  Roswell Park Memorial Institute (cell culture media)
S1P  sphingosine-1-phosphate
S1P1  sphingosine-1-phosphate receptor type 1
SCID  severe combined immunodeficiency
SIV  simian immunodeficiency virus
SLC  secondary lymphoid chemokine
sLeα  sialyl lewis x
SLO  secondary lymphoid organ
SP  CD4 ,CD8 single positive
Spl  spleen
STAT  signal transducer and activator of transcription
Syk  spleen tyrosine kinase
TCP  T cell progenitors
TCR  T cell receptor
TGF  transforming growth factor
Th   T helper
TNF  tumour necrosis factor
VE-cadherin  vascular endothelial cadherin
VCAM-1 vascular cell adhesion molecule 1
VLA-4 very late antigen-4
VLDL very low density lipoproteins
VWF von Willebrand factor
WT  wild-type
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CHAPTER 1: INTRODUCTION

1.1 Overview

The immune system is a complex entity that involves the circulation of leukocytes throughout the body to monitor for disease and infection (von Andrian and Mackay, 2000). It involves strict regulation and control in order to find a homeostatic balance between recognizing harmful foreign pathogens and tolerating self-made antigens. An imbalance to either extreme can lead to infection or to autoimmunity. There are many elements that come together to form the system that tightly controls immune surveillance.

Lymphocytes are an integral part of the immune system and play a critical role in the defence against pathogens. As demonstrated in Severe Combined Immunodeficiency (SCID), Acquired Immune Deficiency Syndrome (AIDS) and other lymphocyte deficiency diseases, lymphocytes are necessary for survival. Without them, the body is plagued with infections that cannot be cleared and healed. They are part of the adaptive immune system and are able to recognize and remember specific pathogens in contrast to leukocytes of the innate immune system that respond to general pathogens but without long term recognition. This allows for a more effective immune response of lymphocytes against both first time and repeat pathogen challenges.

Two types of lymphocytes are B cells which are responsible for secreting antibodies in humoral immunity and T cells which play a critical role in cell-mediated immunity (Janeway, 2005). T cells can further be divided into several different categories. CD4+ T cells, also known as T helper (Th) cells, help activate B cells and macrophages. CD8+ T cells, also known as cytotoxic T cells, kill host cells infected with intracellular pathogens. Regulatory T cells inhibit the activity of other leukocytes thereby suppressing an immune response. Natural killer (NK) T cells recognize glycolipid antigens and secrete cytokines for both cell and humoral mediated
immunity. CD4+ and CD8+ T cells can either be naïve, activated or memory. Naïve T cells have never encountered their cognate antigen and have therefore not been stimulated. Activated T cells, also known as effector T cells, have been stimulated by leukocytes or infected tissues to mount an immune response. Memory T cells develop in the late stages of an immune response and are primed to quickly respond upon re-encountering the pathogen again.

Lymphocytes need to be tightly regulated in their development, their movement around the body, and their function as they have important duties for the survival of an organism but can have disastrous results when they become dysregulated.

1.2 Lymphocyte development

1.2.1 Bone marrow

Lymphocytes originate from haematopoietic stem cells (HSCs) in the bone marrow (Janeway, 2005). HSCs are part of the Lin−Sca-1+ cKit+ (LSK) population. These can either be long-term HSCs or short-term HSCs, indicating their capacity of self renewal. HSCs then differentiate into multipotent progenitors (MPPs) which are no longer capable of self-renewal and are identified by the expression of fms-like tyrosine kinase-3 (Flt3). MPPs further differentiate into early lymphoid progenitors (ELPs) and display a low expression of interleukin (IL) -7 receptor (IL-7R) and recombination activation gene (Rag) -1 which are necessary for B and T cell development. Cells then differentiate into common lymphoid progenitors (CLPs), marked by an increase in IL-7R expression, and at that point, are only capable of giving rise to T, B, and NK cells.

B cells continue their development in the bone marrow to the stage of immature B cell, by which time they have developed a mature B cell receptor (BCR) (Cambier et al., 2007). They finish their development in the peripheral blood and spleen.
1.2.2 Thymus

T cells begin their differentiation process in the bone marrow as lymphoid progenitors but then migrate through the peripheral blood and only become fully mature T cells in the thymus (Chi et al., 2009). The thymus does not contain any cells with self-renewing capacity and therefore relies solely on the immigration of progenitors. There are various types of stem cells found in the blood: HSCs, MPPs, ELPs and CLPs. Another type of progenitor found in the blood, but not in the bone marrow, is the common lymphoid progenitor (CLP) which demonstrate T cell potential, but very little B cell, myeloid or erythroid potential (Kondo et al., 1997). It is still unknown however which of these stem cell populations, if any, are the progenitors that seed the thymus and eventually give rise to mature T cells.

When progenitors make their way from the blood into the thymus, it is believed they enter at the corticomedullary junction. However low numbers of entering progenitors make it difficult to study the homing of cells *in vivo* (Swann and Boehm, 2009). In the perimedullary cortex, one of the earliest intrathymic progenitor subsets is found, the early T lineage progenitor (ETP) which are double negative (DN - lacking both CD4 and CD8 receptors on its surface) (Fowlkes et al., 1985). They go through four stages lacking CD4 and CD8 (DN1-4) and are defined by CD44 and CD25 expression (Godfrey et al., 1993). DN1 cells can be found in the perimedullary cortex for up to ten days and undergo massive proliferation (up to ten serial cell divisions), become committed to the T cells lineage, and then migrate deeper in the cortex (Petrie and Zuniga-Pflucker, 2007; Shortman et al., 1990). DN2 cells migrate towards the subcapsular regions through the inner cortex, taking on average two days, and continue to proliferate. By this point they have already irreversibly committed to the T cell lineage (Masuda et al., 2007). There are two different kinds of T cells that can arise at this point, α:β T cells or γ:δ T cells, defined by the different genetic loci that make up the TCR (Janeway, 2005). γ:δ T cells are less common and little is known about them. For α:β T cells, the β chain genes rearrange
first however if no functional β chain is produced, the cell will not be able to produce a pre-T cell receptor and undergo apoptosis. However, a non-productive β chain can be rearranged again and subsequently rescued. By this point, thymocytes move into the DN3 and DN4 stage and migrate from the inner to outer cortex (Goldsby, 2003). The expression of the pre-T cell receptor and its association with the β-chain will halt β-chain gene rearrangement and stimulate the thymocyte to undergo massive expansion. After proliferation occurs CD4 and CD8 molecules are expressed and thymocytes become double positive (DP – expressing both CD4 and CD8 receptors on its surface) cells. At this point, the α chain undergoes rearrangement.

DP cells make up the majority of the population in the thymus. It is as DP cells that thymocytes undergo the critical process of positive selection. Major histocompatibility complex (MHC)-peptide complexes are expressed by cortical epithelial cells and only thymocytes that are capable of binding their TCR to MHC-peptide will receive a survival signal (Takahama et al.). All other DP cells will die of neglect, accounting for the majority of thymocytes. Cells whose TCR bound MHC class I will develop into CD8+ single positive (SP) T cells and those that bound MHC class II will develop into CD4+ SP T cells while heading to the medulla of the thymus where they undergo negative selection (Nitta et al., 2008). Here they will encounter dendritic cells and macrophages presenting self-antigen. If they bind with overly high affinity, they will receive an apoptotic signal and undergo programmed cell death, thereby preventing self-reacting T cells from exiting the thymus and targeting tissues in the body, leading to autoimmune diseases. Between positive and negative selection, 98% of DP cells will be eliminated, and only 2% will go on to be mature T cells that exit the thymus and released into the bloodstream.

1.3 T cell subsets

After leaving the thymus, mature T cells can be classified in many different ways based upon function, cell surface expression and experience with antigen.
1.3.1 CD4+, CD8+, NK and regulatory T cells

CD4+ T cells are also known as T helper cells (Janeway, 2005). They are able to bind MHC class II molecules which are found on antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells. CD4+ T cells function as activators of other immune cells. Depending on external cues, they can develop into Th1, Th2 or Th17 cells which can be categorized by the cytokines they release and the leukocytes they activate. This differentiation will determine whether the immune response will be cell mediated or humoral mediated.

Viruses and some bacteria induce dendritic cells to secrete IL-12 and interferon (IFN) -α which stimulate the cells to develop into Th1 cells. Th1 cells produce IL-2, IFN-γ, tumour necrosis factor (TNF) –β which stimulate macrophages and CD8+ T cells for a cell-mediated immune response. IL-2 also stimulates Th1 cells to proliferate in an autocrine type fashion. Other pathogens, such as worms, stimulate the production of IL-4 by APCs which causes CD4+ T cells to develop into Th2 cells. These cells in turn produce IL-4, IL-13 and IL-5 as well as stimulate B cells to activate and secrete antibodies. This mediates the humoral immune response. Lastly, Th17 cells have been recently discovered and produce IL-17 which in turn stimulates granulocytes. Transforming growth factor (TGF) -β, IL-1β, IL-6, IL-21 and IL-23 have all been implicated in the differentiation of Th17 cells (Dong, 2008).

CD8+ T cells are also known as cytotoxic T cells. They bind to MHC class I molecules which are expressed on almost every cell in the body. Cells are continually presenting peptides on their surfaces for monitoring by T cells. Should a cell be infected by a foreign pathogen, it will present foreign peptides that may be recognized by CD8+ T cells. This will cause the CD8+ T cell to release cytotoxins that form pores in the cell membrane of the infected cell, inject granzymes and activate apoptosis through caspases. CD8+ T cells can also activate the Fas pathway in infected cells which also leads to apoptosis.
NK T cells share characteristics of both NK cells as well as T cells, namely expressing CD161, or NK1.1, normally expressed on NK cells, as well as CD3, the TCR found on T cells (Godfrey et al., 2010). NK T cells may either express CD4, CD8 or neither. These cells recognize CD1d, an antigen presenting molecule that binds self and foreign lipids. They can promote cell-mediated immunity to tumours as well as infectious organisms.

Regulatory T cells are very unique in that instead of mounting an immune response, they suppress the activity of other leukocytes (Thompson and Powrie, 2004). They can develop in the thymus (natural regulatory T cells) as well as be converted from stimulated T cells in the periphery (inducible regulatory T cells). Regulatory T cells are critical for the maintenance of self tolerance by regulating potential autoreactive immune effectors as well as preventing immune hypersensitivity reactions in allergies by suppressing proinflammatory responses.

1.3.2 Naïve, effector and memory T cells

Naïve T cells are those that have not yet encountered their cognate antigen. They are defined as L-selectin\textsuperscript{high} which is needed for entry into secondary lymphoid organs (SLOs). SLOs consist of lymph nodes, spleen, Peyer’s patches and other mucosal associated lymphoid tissues like tonsils, adenoids and appendix (Janeway, 2005). Naïve T cells circulate through the body via blood and lymph to various SLOs in search of signs of infection that may be recognized by their specific TCR.

Effector T cells are those that have recognized their cognate antigen in SLOs by an APC. They shed L-selectin and downregulate C-C chemokine receptor type 7 (CCR7) on their cell surface as well as upregulate the activation markers CD69 and CD25. They expand prolifically in the SLOs before migrating out into the blood in search of the area of infection. There they target infected cells for apoptosis and aid other leukocytes in mounting an immune response in attempts to clear the area of invading pathogens.
Memory T cells are formed following a mounted immune response against a foreign pathogen. Upon antigen contact, effector cells are generated along with early memory T cells, all of which die off after the pathogen has been removed, with the exception of a small number of long lived memory T cells which monitor and protect against recurrence of infection. They are often defined as CD44$^{\text{high}}$ cells in mice, or CD45RO$^{\text{high}}$ cells in humans, and can be classified into two categories, central memory T cells, L-selectin$^{\text{high}}$ CCR7$^{\text{high}}$, and effector memory T cells, L-selectin$^{\text{low}}$ CCR7$^{\text{low}}$ (Sallusto et al., 1999). As L-selectin and CCR7 are needed for entry into SLOs, central memory T cells are destined to travel from SLO to SLO looking for signs of reinfection of the original pathogen that stimulated its cognate antigen. Effector memory T cells do not express L-selectin and CCR7 but have high levels of $\beta$-1 and $\beta$-2 integrins, suggesting they are capable of quickly entering inflamed tissues and perhaps reside there although effector memory T cells can be found in SLOs as well. Effector memory T cells can be directly stimulated in non-lymphoid tissues by resident dendritic cells and do not require being in lymphoid tissues as is the case for initial activation of naïve T cells upon antigen stimulation (Wakim et al., 2008). Memory T cells are capable of mounting a secondary immune response much quicker than naïve T cells in a primary immune response to a pathogen. Effector memory T cells however expand into effector cells after antigen detection much more rapidly than central memory T cells.

The regulation of T cells is critical and each subset has varying requirements for activation, regulation and maintenance. While they are important for removing foreign pathogens, they are also capable of mistakenly targeting the host itself which is why it is important that there are many mechanisms in place to regulate the homeostasis of T cells in the periphery.
1.4 Homeostasis

"Homeostasis is defined as the ability of a biological system to maintain internal equilibrium by adjusting its physiological properties" (Baccala and Theofilopoulos, 2005). The process of leukocyte regulation and survival is very dynamic and complex. T cells are long-lived cells that rarely divide and continuously circulate through blood and SLOs monitoring for indications of infection. T cells are continuously being produced by the thymus and released into the periphery and yet the number of T cells in the blood and lymph nodes under non-inflammatory conditions remains constant over time. This suggests they disappear at the same rate they are being produced in the thymus. Within the early stages of life, the T cell population tends to consist mostly of naïve T cells with only 10-20% memory T cells (Sprent et al., 2008). However, as time passes and the body encounters antigens that stimulate the immune system, the peripheral T cell population shifts to memory T cells. Also, the thymus atrophies after the second decade of life in humans emitting fewer and fewer naïve T cells as time passes while the cellularity of the lymph nodes and spleen remains relatively constant. Despite the reduction in cell output, the body maintains constant levels of T cells which are controlled by complex homeostatic mechanisms.

1.4.1 Naïve T cells

Naïve T cells are resting cells that circulate through the body and rarely, if ever, divide. In mice they are often defined as CD44\textsuperscript{low} cells, in humans they are defined as CD45RO\textsuperscript{low}, but they also express high levels of L-selectin and CCR7 necessary for migration into lymph nodes. They have gone through a rigorous selection process in the thymus in which they are tested against dangerously high affinity for self ligands as well as having a weak affinity for the MHC-self peptide complexes, termed negative and positive selection, respectively. During positive selection, a thymocyte receives a signal after its TCR weakly binds MHC that rescues it from death by neglect. After leaving the thymus and entering the circulation, T cells continue to
require the signals from MHC-TCR binding in the periphery to maintain viability (Takeda et al., 1996; Tanchot et al., 1997). They also require IL-7 since T cells in an IL-7-depleted environment are not viable (Schluns et al., 2000; Tan et al., 2001).

### 1.4.2 Memory T cells

Memory T cells also circulate through the body monitoring for disease, but have a more rapid turnover rate than naïve T cells, dividing once every 2-3 weeks (Tough and Sprent, 1994). Interestingly, young adult mice that are housed in pathogen-free environments have similar numbers of memory T cells compared to mice housed in conventional environments, suggesting that memory T cells can also be formed against self antigens (Min et al., 2005; Pereira et al., 1986). This suggests there are two types of memory cells, traditional memory T cells that are formed after antigen encounter and mounted immune response, and memory phenotype T cells that are formed after encountering self antigen which causes expansion but bypasses the activation and effector stage. In contrast to naïve T cells, when memory T cells are transferred to MHC deficient hosts they survive for prolonged periods suggesting maintenance of memory T cells does not require MHC-TCR interaction (Murali-Krishna et al., 1999; Swain et al., 1999).

There is a small population of CD8+ memory T cells that requires MHC contact and they all contain a semi-activated phenotype. However, the majority of CD4+ and CD8+ memory T cells do not require MHC stimulation for viability in vivo (Boyman et al., 2006a; Murali-Krishna et al., 1999; Swain et al., 1999). Memory T cells require interactions with cytokines IL-7 and IL-15 for survival (Judge et al., 2002; Purton et al., 2007). IL-15null mice contain very few CD8+ memory T cells, and those that do exist show a semi-activated state. However, CD4+ memory T cell numbers are unaffected in IL-15null mice suggesting it is only important for CD8+ memory T cell survival (Judge et al., 2002; Kennedy et al., 2000). IL-7 was determined to be important for both CD4+ and CD8+ memory T cells since memory T cells are not viable in IL-7null mice (Kondrack et al., 2003) (Carrio et al., 2007). IL-7 and IL-15 are found in low quantities under normal
conditions and both are needed for CD8+ memory T cell survival. However, if IL-7 levels are increased in a transgenic mouse model, CD8+ memory T cell numbers increase even if IL-15 is not present (Kieper et al., 2002).

1.4.3 Sites of homeostatic signals

It is hypothesized that since naïve T cells continuously circulate through the body monitoring for foreign antigen in the lymph nodes, it is likely they also receive their survival signals in the lymph nodes as opposed to in the blood or lymph while in transit (Surh and Sprent, 2008). Indeed, mice lacking SLOs had impaired survival of naïve T cells, particularly CD4+ T cells (Dai and Lakkis, 2001). IL-7 is produced by stromal and epithelial cells in the bone marrow and thymus, and by fibroblastic reticular cells (FRCs) in lymph nodes (Goodwin et al., 1989; Link et al., 2007; Namen et al., 1988). IL-7 would likely be displayed at the site of production, bound to the extracellular matrix, as opposed to being secreted in soluble form. CD4+ and CD8+ memory T cells have been shown to also migrate to and from the bone marrow, another source of IL-7, and may receive their survival signals there as well. CD4+ effector memory T cells expressing high levels of Ly6c have been shown to predominantly home to the bone marrow, whereas the spleen contains mainly Ly6c<sup>low</sup> effector memory T cells (Tokoyoda et al., 2009). CD4+ memory T cells in the bone marrow have been shown to be in direct contact with IL-7-expressing stroma cells. Lymph nodes and bone marrow are also a rich source of dendritic cells, which presents MHC-II, required for T cell survival of CD4+ T cells, as well as MHC-I for survival of CD8+ T cells. Interactions of T cells with dendritic cells in the blood or lymph are less likely to occur considering the shear flow rate (Di Rosa and Pabst, 2005).

1.4.4 Cytokines in T cell homeostasis

Cytokines are small soluble proteins that are secreted by cells to influence the behaviour of surrounding cells or itself (Goldsby, 2003). There are a number of cytokines that are important for maintenance of both naïve and memory T cells. IL-7 plays an important role both
for the development of T cells in the thymus as well as their survival in the periphery. IL-7 production is not affected by external stimuli, but rather, levels of IL-7 are determined by the rate that it is scavenged by T cells. It is believed that cells that receive signals from IL-7 promptly downregulate and suppress the IL-7Rα chain (CD127) thereby ensuring the maximum amount of T cells will benefit from the IL-7 that is present, receiving prosurvival signals (Park et al., 2004). This explains why there is such a broad range of IL-7Rα expression by naïve T cell populations in the lymph nodes. IL-7 is recognized by the IL-7R which consists of the IL-7Rα-chain and the common cytokine-receptor γ-chain (CD132). The common γ chain (γc) is shared among receptors that recognize a family of cytokines consisting of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Figure 1.1). The heterotrimer receptors IL-2 and IL-15 also share the IL-2Rβ (CD122) chain in addition to the γc and therefore have been shown to have many overlapping functions. These two subunits are important in signalling. IL-15Rα (CD215) is structurally related to IL-2Rα (CD25) but is capable of binding IL-15 with a 1000 fold high affinity than IL-2 binding with IL-2Rα (Giri et al., 1995). At high concentrations though, both IL-15 and IL-2 are capable of stimulating signalling through the β and γ chains without the α chain present (Carson et al., 1994; Giri et al., 1994; Pitton et al., 1993). For IL-2 signalling, the IL-2Rα subunit is not essential but mediates high-affinity binding of the cytokine (Willerford et al., 1995). High affinity αβγ IL-2Rs are generally found on CD4+ regulatory T cells and recently activated T cells. High affinity αβγ IL-15Rs are found at a low level on naïve CD8+ T cells and in abundance on memory CD8+ T cells. Low affinity βγ receptors, which are the two common subunits for both IL-15 and IL-2, are also found at a low level on naïve CD8+ T cells and at high levels on memory and memory-phenotype CD8+ T cells as well as NK cells (Letourneau et al., 2009; Malek, 2008). All the members of the γc family induce signalling through the janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (Rochman et al., 2009). IL-7R signalling promotes cell survival by regulating the B cell lymphoma 2 (Bcl-2) family. The anti-apoptotic signalling molecules Bcl-2 and induced myeloid
leukemia cell differentiation protein (Mcl-1) are induced, whereas the pro-apoptotic signalling molecules Bid, Bim and Bad are inhibited upon IL-7 binding (Jiang et al., 2005). Overexpression of Bcl-2 or knocking out the Bim gene in IL-7R-deficient mice partially restores the number of thymocytes and peripheral blood T cells (Akashi et al., 1997; Pellegrini et al., 2004). There are various other cytokines and chemokines that have been implicated to have a role in the survival of T cells including IL-2, IL-21, CCL19, and Chemokine (C-X-C motif) ligand 12 (CXCL12) (Ferrari-Lacraz et al., 2008; Kelly et al., 2002; Link et al., 2007; Suzuki et al., 2001).
1.4.5 Homeostatic proliferation and lymphopenia

The number of T cells in the periphery remains relatively constant over time. Despite T cell output from the thymus gradually decreasing, as well as sudden temporary drops in T cell numbers by things like cytotoxic drugs, irradiation or certain viruses, such as human immunodeficiency virus (HIV), the body has mechanisms to expand the number of T cells back to constant levels. The reduction of lymphocytes in the periphery is termed lymphopenia. When T cells are severely reduced, the body will compensate by causing circulating T cells to proliferate and fill the T cell compartment back to normal levels in a process known as homeostatic proliferation, or lymphopenia induced proliferation (LIP) (Ernst et al., 1999). The mechanism for controlling the number of T cells in the periphery has been attributed to limiting amounts of survival factors such as cytokines as well as TCR stimulation (Surh and Sprent, 2008). Therefore, the same factors that are important in the maintenance and survival of T cells under normal homeostatic conditions are also responsible for proliferation of these cells under lymphopenic conditions. Homeostatic proliferation is most often studied in models such as TCR\(^{-/-}\), Rag\(^{-/-}\), IL-7R\(^{null}\), sub-lethally irradiated, nude or SCID mice due to their severe lymphopenia. Adoptive transfer of wild-type (WT) T cells in these hosts show substantial proliferation (Surh and Sprent, 2008). T cells that undergo proliferation under these conditions upregulate cell surface levels of CD44, just as memory T cells do, hence the term memory phenotype cells. The process of homeostatic proliferation allows for a maximum diversification of T cell repertoire over time while also maintaining control over T cell populations so that they do not proliferate out of control, nor dwindle to too few numbers.

1.4.5.1 Role of IL-7 in homeostatic proliferation

When T cell numbers in the periphery decline, the consumption of IL-7 declines as well causing an increase in background levels of IL-7. Under these circumstances, naïve T cells begin respond to self antigens, come out of interphase and slowly begin to divide. This process
also requires TCR/MHC interaction along with increased levels of IL-7. They will continue to slowly divide until approximate normal T cell levels are reached. CD8+ T cells are more responsive to homeostatic proliferation than CD4+ T cells, one possible reason being that high levels of IL-7 in lymphopenic mice will cause a decrease in MHC-Class II expression on the surface of dendritic cells, thereby limiting their interaction with CD4+ T cells and preventing expansion (Guimond et al., 2009). Homeostatic proliferation is typically a slow process in which most cells only divide 1-5 times over a period of 1-2 weeks (Sprent et al., 2008). This may be to limit proliferation during temporary states of lymphopenia, for example after infection with the common cold virus, or corticosteroid use so that proliferation only occurs under chronic states of lymphopenia, for example after full body radiation or chemotherapy. This differs from division caused by activation which happens much more rapidly. Upon stimulation by foreign antigen, naïve T cells turn into effector cells and undergo massive proliferation followed by wide scale death. Activation also requires costimulation by B7-CD28 and CD40-CD40L interactions whereas homeostatic proliferation does not (Prlic et al., 2001). It only requires IL-7 and MHC stimulation. There is a small population of cells that are capable of proliferating rapidly when IL-7 is not present, but this is costimulation dependent. It appears that this is induced by commensal bacteria since the effect is much less pronounced in germ-free hosts (Kieper et al., 2005). Upon division, homeostatically proliferating T cells acquire functional and phenotypic features of memory T cells such as high levels of CD44 expression and respond to foreign antigen similarly to central memory T cells (Goldrath et al., 2000; Haluszczak et al., 2009). Homeostatic proliferation in naïve and memory T cells can also be induced in non-lymphopenic animals by increasing levels of IL-7 with IL-7/antibody complexes (Boyman et al., 2008).

As previously mentioned, young adult mice show 10-20% memory phenotype T cells despite having limited encounters with foreign pathogens. In young mice, T cells with a memory phenotype can be detected within the first few weeks of life with up to 50% of circulating cells
being CD4\textsuperscript{high}. This is attributed to IL-7 induced homeostatic proliferation. As T cells start emerging from the thymus, they enter an IL-7 elevated environment and homeostatically expand, creating memory phenotype T cells (Ichii et al., 2002). It is speculated that these original cells may account for the number of memory T cells found in young adults despite having limited exposure to pathogens.

1.4.5.2 Role of TCR stimulation in homeostatic proliferation

Homeostatic proliferation also requires TCR stimulation by MHC in naïve cells similarly to the way it requires stimulation in steady state survival (Beutner and MacDonald, 1998; Ernst et al., 1999). However, it does play a role in various other ways. Interestingly, not all naïve T cells are able to efficiently undergo homeostatic proliferation. Studies with polyclonal T cells show that only approximately 30% of the population of naïve CD4+ and CD8+ T cells are able to expand under these circumstances (Ernst et al., 1999). T cells with high-affinity binding TCRs show a preference for homeostatic proliferation in comparison to T cells with a low-affinity binding (Kieper et al., 2004). This is demonstrated in certain transgenic lines. OT-I, 2C, P14, DO11, 1H3.1 are able to homeostatically proliferate whereas OT-II and HY are not as they have below-average TCR affinity (Surh and Sprent, 2000). To support this, T cells that are CD5\textsuperscript{high} show superior capability to homeostatically expand (Kieper et al., 2004). CD5 is a negative regulator of TCR and BCR signalling and therefore its expression levels correlate with signalling intensity; high levels of CD5 indicate an increase in TCR suppression, therefore, suggest strong TCR affinity and signalling (Azzam et al., 2001). Interestingly, despite there being a preference for select T cells during homeostatic proliferation, there is still diversity in the T cell pool as the T cell repertoire does not overly skew in favour T cells with a high affinity TCR. This can be attributed to specific competition between T cells, called intraclonal competition, suggesting that T cells of the same MHC specificity must compete for the same set of self-peptide-MHC complexes, whereas T cells of different MHC specificity in the same environment do not
compete and therefore thrive equally as well (Leitao et al., 2009; Min et al., 2004; Moses et al., 2003; Troy and Shen, 2003). In addition, recent studies, while still controversial, have shown that depriving CD8+ T cells of MHC-I interaction causes them to be more responsive to low-affinity peptide-MHC ligands in a non-lymphopenic setting, suggesting that the requirement of high-affinity interactions may be caused by lymphopenic environments (Takada and Jameson, 2009).

1.4.5.3 Role of other cytokines in homeostatic proliferation

While IL-7 appears to be the dominant cytokine involved in homeostatic proliferation in vivo, other cytokines have been implicated to play a role. IL-2 and IL-15 are also able to induce strong proliferation of T cells when found in excess amounts. However, proliferation does not occur if IL-7 is not present, regardless of the amount of IL-2 or IL-15 available (Ge et al., 2002; Tan et al., 2001; Wherry et al., 2002). IL-2, IL-7 and IL-4 stimulated proliferation is much more pronounced when complexed with anti-IL-2, IL-7 or IL-4 monoclonal antibody (mAb) respectively (Boyman et al., 2006b; Kamimura et al., 2004). This process is also MHC-dependent since proliferation is undetectable in MHC−/− mice (Cho et al., 2007). Interestingly though, if large quantities of WT cells are transferred into MHC−/− mice, the MHC on the donor T cells are capable of stimulating the TCR on neighbouring donor T cells, causing proliferation.

Cytokines affect naïve and memory T cells differently. Memory T cells express high levels of IL-2Rβ, a receptor chain that binds both IL-2 and IL-15, and therefore proliferate more rapidly than naïve T cells that express much lower levels of IL-2Rβ. When in the presence of memory T cells, naïve T cells will divide poorly because IL-2 and IL-15 will be consumed by the memory T cells. However, if memory T cells are not present, naïve T cells are capable of dividing efficiently (Cho et al., 2007; Kamimura and Bevan, 2007). While CD8+ naïve T cells have low IL-2Rβ expression, CD4+ naïve T cells have almost undetectable levels which may explain why CD8+ T cells are much more responsive than CD4+ T cells (Zhang et al., 1998).
This is also the case for memory T cells. CD4+ memory T cells have lower levels of IL-2Rβ than CD8+ memory T cells and therefore IL-2 and IL-15 have a greater effect on CD8+ memory T cells. IL-15 also works in combination with IL-21, another γc cytokine. The combination of these two cytokines in vitro induced a synergistic proliferative effect of naïve and memory CD8+ T cells as well as an increase in IFN-γ production and in vivo, proliferation of CD28+ CD8+ memory T cells (Nguyen and Weng, 2010; Zeng et al., 2005).

Homeostatic proliferation of naïve CD8+ T cells initially occurs in the lymph nodes, but not in spleen, and requires L-selectin for lymph node entry (Schuster et al., 2009). CD8+ memory T cells prefer the bone marrow as their site of homeostatic proliferation (Becker et al., 2005). Consistent with these observations, cytokines and APCs needed in homeostatic proliferation can be found in both these locations.

1.4.5.4 Autoimmunity in homeostatic proliferation

Homeostatic proliferation, while important for the survival of an organism after the severe reduction of T cells, can also cause problems with autoimmunity. T cells with high affinity TCR receptors, being tickled by self ligands, sometimes expand out of control, bypassing control mechanisms, and begin attacking the body itself. Lymphopenia has been associated with the autoimmune disorders Type 1 diabetes, rheumatoid arthritis, Sjogren’s syndrome, and lupus in humans. In rodents, it has been associated with diabetes and inflammatory bowel disease (Baccala and Theofilopoulos, 2005). One mechanism in place to prevent uncontrolled homeostatic proliferation that may cause autoimmunity is regulatory T cells. It was shown that they selectively inhibit homeostatic proliferation, which also allows the maintenance of TCR diversity (Winstead et al., 2010). When Foxp3+ regulatory T cells were absent during homeostatic proliferation and reconstitution, there were “holes” in the TCR repertoire, and a loss of antigen-specific responsiveness to particular microorganisms, whereas in the presence of regulatory T cells, optimal TCR diversity and foreign antigen responsiveness was demonstrated.
Clinical implications of homeostatic proliferation

Homeostatic proliferation and general T cell survival has been a subject of interest for human therapies. Decreased numbers of T cells can be caused by many factors, including viruses, chemotherapy cancer treatment, bone marrow transplant irradiation and general aging. A method to increase T cell numbers safely and within the own body is thus highly desirable. Recent phase I clinical trials treating cancer patients with recombinant human IL-7 resulted in successful immune reconstitution showing increased numbers of naïve and central memory T cells and a large TCR diversity (Sportes et al., 2008). The administration of recombinant human IL-2 has been extensively studied in subjects infected with HIV, but has not been successful in boosting T cells numbers in HIV infected patients (Della Chiara et al., 2010). rhIL-7 has recently come out of preliminary phase I trials and seems to be having promising results with increased T cell counts in HIV patients (Levy et al., 2009; Sereti et al., 2009). IL-7 has also been tested in macaques infected with simian immunodeficiency virus (SIV) and has shown consistent but transient increases in peripheral blood T cells (Beq et al., 2006; Parker et al., 2010). Sportes, et al. suggest that the resulting circulating T cell pool after rhIL-7 treatment closely resembles that seen earlier in life (Sportes et al., 2008). Therefore, if IL-7 or other homeostatic proliferation therapies prove effective, it may open up the possibility of eliminating age related illness caused by an ineffective immune system. Homeostatic proliferative therapies would also be very beneficial to recipients of bone marrow transplants. Currently, patients require weeks of recovery for leukocytes to repopulate after transplantation, leaving them susceptible to infection. The ability to expand T cells may shorten the window of susceptibility in patients. These therapies would have to be closely regulated to prevent autoimmunity caused by homeostatic expansion, for example by ensuring that all T cell subsets, particularly T regulatory cells remain in balance.
1.5 T cell migration

While T cells use the blood as a transport system to move around the body, they do not remain in the circulation for long. They enter SLOs where they will receive survival signals from resident cells and the cytokines they secrete, as well as search for indications of infection. On average, naïve T cells only spend 30 minutes in the circulation before entering another SLO (Pabst, 1988). It is suggested that 1 in every 4 circulating lymphocytes will leave the blood after entering the vasculature of an SLO (Hay and Hobbs, 1977). It is believed all T cells remain in continuous circulation and do not reside in any one location for long. This was demonstrated by continually draining lymph from the thoracic duct, which causes the entire body to be depleted of naïve lymphocytes (Gesner and Gowans, 1962). The movement of cells from the blood to the tissues is a complex process. The blood is pumped through the blood vessels with considerable force and T cells are required to leave the fast moving system at precise locations. The arrest of T cells and their migration through the blood vessel wall into underlying tissues is a sophisticated and coordinated interplay controlled by various adhesion and signalling molecules. The mechanism controlling lymphocyte entry into lymph nodes, an adhesion cascade, is also found in other locations of the body, such as progenitors entering the thymus and activated leukocytes entering inflamed tissues. While the paradigm remains the same, they all use different adhesion molecules and chemokines, but have similar functions and behaviours (Figure 1.2).

The adhesion cascade includes several steps that have been characterized as rolling, cell activation, adhesion and transmigration. However, recent, more detailed characterization of the molecular mechanisms behind the homing process show a number of intermediate steps. A recent review suggests that slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration and migration through the basement membrane are also
Lymphocyte homing to SLOs

Progenitor homing to thymus

T cell recruitment to inflammation
Figure 1.2: The adhesion cascade. Cells use the same mechanisms to exit the blood and into tissues but use different adhesion molecules and chemokines. In this simplified diagram, lymphocyte movement into lymph nodes through HEVs requires L-selectin on lymphocytes to bind to PNAdS on the endothelial cells of the blood vessels causing rolling before binding to integrins and firmly adhering. CCL21, CCL19 and CXCL12 are involved in attracting lymphocytes to HEVs as well as triggering activation of integrins from a semi-active to an active state. Once adhered, lymphocytes transmigrate between or through the endothelium of the blood vessels. In progenitor homing to the thymus, PSGL-1 on the progenitors and P-selectin on the endothelial cells are responsible for rolling on the blood vessel walls. In activated leukocyte homing to inflamed tissues, PSGL-1 on progenitors and P-selectin and E-selectin on inflamed endothelial cells mediate rolling. Differing chemokines and integrin patterns allow each situation to have unique selectins and integrins to mediate lymphocyte entry into tissues.

steps that need to be included to the original four steps in the adhesion cascade to make it more comprehensive (Ley et al., 2007).

In general, chemoattractants are released in the blood creating a gradient to attract migrating cells. The chemoattractants also induce signalling to prepare cells for rolling and arrest and transmigration. Once in the area of their destination, selectins and their ligands will interact in a low affinity manner, allowing for brief tethering causing cells to roll along the surface of the blood vessel wall. The reduction in speed also allows for better interaction with chemokines. Chemokine binding to migrating cells activates other mediators such as integrins which have a higher affinity bond with intercellular adhesion molecules (ICAMs) that cause cell arrest. While stably fixated on the wall of the blood vessel, cells are able to penetrate through the endothelium to reach the tissues that lie beneath.

1.5.1 Naïve T cell entry in lymph nodes

Lymphocytes travel through the blood until they reach a lymph node. Adhesion molecules are absent in lymph node arterioles and capillaries but they frequently found in venules (von Andrian and Mempel, 2003). Naïve T cells traveling through the blood have high expression levels of L-selectin on their surface, a cell adhesion molecule with low affinity binding
capabilities. L-selectin interacts with peripheral node addressins (PNAds) found on the surface of the blood vessels in the lymph nodes. PNAds represent a group of L-selectin ligands including glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), CD34, podocalyxin, endoglycan, endomucin and nepmucin, all consisting of a protein backbone decorated with the specific O-linked oligosaccharide 6-sulfo sLe\(^x\). PNAds are found on high endothelial venules (HEVs) which consist of a specialized type of cuboidal endothelial cell that is surrounded by an unusually thick basal lamina located in the upstream portion of the post-capillary venules of SLOs, with the exception of spleen (Tohya et al., 2010). These cells are specialized to express important adhesion molecules for lymphocyte homing as well as for efficient transmigration. They have a broad distribution of PNAds along the HEV lumen which allows for rolling of lymphocytes along the entire luminal surface of HEVs through the interaction with L-selectin which is found on the tips of microvilli of lymphocytes allowing for maximum interaction between lymphocyte and endothelial wall. This interaction is of low affinity and therefore the binding partners catch briefly then release. This repeated action accounts for the rolling action of lymphocytes on the HEVs. While slowed down, lymphocytes have the opportunity to interact with other local adhesion molecules and chemokines. Lymphocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4) are integrins found on the surface of T cells that are generally kept in a low affinity, non-extended state (McEver and Zhu, 2007). LFA-1 binds ICAM-1 and ICAM-2 while VLA-4 binds vascular cell adhesion molecule-1 (VCAM-1) found on endothelial cells (Goldsby, 2003). Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is another molecule that can bind L-selectin and integrins but is only found in mesenteric lymph nodes, Peyer’s patches and other gut associated lymph tissues. The chemokines Chemokine (C-C motif) ligand 21 (CCL21) and CCL19 are presented on the HEVs and interact with CCR7 on the T cells, signalling to transiently activate LFA-1 and VLA-4 to an extended state. Then, the shear forces from the flow of the circulatory system stabilize the extended LFA-1 and VLA-4 high affinity conformation for ICAM-1 and VCAM-1 respectively, causing cell arrest. Soluble
chemokine stimulation alone is not sufficient to activate integrins but require shear flow to make them adhesive (Woolf et al., 2007). This may explain why lymphocytes are also able to migrate toward CCL21 and CCL19 while in lymph nodes but do not adhere to stromal cells since there is no shear flow to cause arrest of cells. Under in vitro conditions, CXCL12 has been shown to mediate T cell adhesion to ICAM-1 through activation of LFA-1. The effect, however, is less evident in vivo (Campbell et al., 1998; Okada et al., 2002). CXCL12 may play a similar role to CCL21 and CCL19 but this is only seen in their absence, suggesting that CCL21 and CCL19 are the primary chemokines involved in this process and CXCL12 is only secondary.

After stable adhesion to the blood vessel wall, T cells will seek out a preferred site of transmigration where there is minimal disruption to the vessel walls (Ley et al., 2007). This process is referred to by various names including transmigration, transendothelial migration, extravasation and diapedesis and is primarily mediated by ICAM-1 on endothelial cells and LFA-1 on T cells. It is generally believed that lymphocytes are capable of migrating either through a paracellular pathway, whereby lymphocytes migrate between open interendothelial junctions regulated by adhesion molecules induced by cytokines and through the local destruction of the vascular endothelial (VE)-cadherin complex, or through the transcellular pathway, in which lymphocytes will pass through an individual endothelial cell triggered by redistribution of the actin cytoskeleton, F-actin microfilaments and other proteins of the endothelial cell (Carman et al., 2003; Nieminen et al., 2006). Cells must pass through the endothelial cells, the endothelial-cell basement membrane and pericytes before reaching the paracortex of the lymph node (Ley et al., 2007).

1.5.2 T cells in the lymph nodes

Lymph nodes are highly organized, densely packed organs that are extremely important for immune monitoring. The body is too large for each individual lymphocyte to travel every region to monitor for infections, and therefore, immune cells are designed to come together and
interact in lymph nodes and other SLOs and communicate to one another if there is a foreign pathogen. Lymph nodes consist of three main regions, the cortex, the paracortex and the medulla (Goldsby, 2003). The outermost layer, the cortex, consists mainly of B cells, macrophages and follicular dendritic cells arranged in primary follicles. After antigen challenge, the primary follicles will enlarge into secondary follicles and germinal centres. The paracortex, found beneath the cortex, consists mostly of T cells and activated dendritic cells expressing high levels of MHC class II molecules. The medulla consists of two structures, the medullary cords which are strings of macrophages, plasma cells and memory T cells and the medullary sinuses containing histiocytes (immobile macrophages) and reticular cells. The paracortex is also arranged in paracortical cords that originate close to the follicles and extend towards the medulla where they merge into medullary cords (Kelly, 1975). Paracortical sinuses, like medullary sinuses, are lymph-filled spaces bordering the cords and are permeated by reticular fibres. Each paracortical cord has an HEV that is surrounded by FRCs, a type of pericyte. It is believed that lymphocytes migrate along the networks of FRCs that can be seen arranged in spiral layers around the HEVs of around 10-15 µm corridors (Gretz et al., 1997). After migrating from the blood through the HEVs, lymphocytes find themselves in the paracortex where T cells will remain and B cells will migrate to the follicles.

In addition to entry from peripheral blood, lymphocytes are also able to enter the lymph nodes through another route, the lymphatics. The lymphatic system is a circulatory system consisting of a network of conduits that carry lymph from tissues, through SLOs and then returning its contents back into the blood (Janeway, 2005). Lymph enters from the afferent lymph vessels into the subcapsular sinus, a hollow space found underneath the fibrous capsule that surrounds the lymph node (von Andrian and Mempel, 2003). The leukocytes then travel through the trabecular sinuses toward the cortical then medullary sinuses. They are not, however, able to pass into the cortical lymphocyte compartment (Gretz et al., 2000). Only small
molecules with a molecular radius of 4 nm are capable of passing through the FRC conduit such as chemokines and antibodies, but not leukocytes. Memory lymphocytes often use this route to enter lymph nodes, but naïve T and B cells prefer to enter lymph nodes via the blood (Marchesi and Gowans, 1964).

T cells migrate to SLOs for two overlapping reasons. First, to monitor for signs of infection and second, to receive homeostatic cues needed for lymphocyte maintenance and regulation. Dendritic cells are professional APCs that interact with lymphocytes, presenting foreign antigens that they have captured and processed. They can either have traveled from other areas of the body where they would have picked up local antigens, or be resident dendritic cells in the lymph nodes attached to the FRC conduit that sample the lymph content for foreign proteins (Banchereau et al., 2000; Sixt et al., 2005). Lymphocytes then scan dendritic cells, searching for those bearing a matching antigen to its TCR. T cells use the FRC network as a guide for efficient movement through the densely packed lymph node. When T cells leave the blood, traveling through the HEV, they immediately associate with the FRC network and crawl along the strands and presumably mediate their interaction with dendritic cells that are colocalized to the same network (Bajenoff et al., 2006). The FRC network defines the T cell zone and therefore the restriction of T cells to this network prevents T cells from migrating to other areas of the lymph nodes. FRCs express adhesion molecules and present chemokines that support T cell movement. CCL21 and CCL19 are instrumental in guiding T cells throughout the cortical areas (Okada and Cyster, 2007; Worbs et al., 2007).

As mentioned, T cells have a second purpose for spending time in the lymph nodes, to receive homeostatic cues needed for maintenance and functional regulation which is discussed in depth in the Homeostasis section of this thesis. In short, T cells require cytokine and TCR stimulating signals to remain viable and prevent apoptosis. FRCs produce IL-7 and CCL19 which are known naïve T cell survival factors and are thought to be deposited along the FRC.
fibres (Link et al., 2007). Therefore, T cells traveling along the FRC conduit will encounter dendritic cells to scan for signs of infection as well as receive survival signals.

1.5.3 T cell egress from lymph nodes

T cells will spend 8-12 hours in a SLO scanning dendritic cells for cognate antigen before exiting and moving on to the next SLO (Schwab and Cyster, 2007). The process of T cell egress is much less understood than T cell entry. However, there have been numerous recent studies that are starting to elucidate the process. It is believed that egress from lymph nodes occurs at the medullary sinuses connected to the subcapsular space and efferent lymphatic, but it is not yet firmly established (Sanna et al., 2006; Wei et al., 2005). Sphingosine-1-phosphate (S1P) is a sphingolipid that mediates the movement of T cells out of the lymph nodes. It is an important cellular metabolite part of the sphingomyelin cycle, an intermediate in the degradation of sphingolipids (Rivera et al., 2008). It acts as a signalling molecule. It has five known receptors, S1P receptor type 1 (S1P₁) being the one preferentially expressed on lymphocytes. T cells lacking S1P₁ are incapable of exiting the thymus and SLOs into peripheral blood (Allende et al., 2004; Matloubian et al., 2004). Mature B cells also require S1P₁ expression to leave SLOs, but not to leave bone marrow after maturation. Immature B cells, T cells and stem cells, however, do require S1P₁ to exit bone marrow (Allende et al., 2010; Maeda et al., 2010; Massberg and von Andrian, 2009). Expression of S1P₁ is very sensitive to S1P presence and is quickly downregulated upon contact (Schwab et al., 2005). Therefore, S1P₁ surface expression levels are undetectable on T cells in the blood whereas T cells in SLOs show high levels of S1P₁. S1P is present in a gradient where the highest concentration is found in the blood, ranging around 2-3 µM. The lymph has less S1P than the blood and the lymph nodes have even less than the lymph, ranging approximately a thousand fold less than the blood (Gossens et al., 2009; Pappu et al., 2007). S1P is primarily produced by red blood cells, although also by platelets, mast cells and other leukocytes, and also by vascular and lymphatic endothelial cells.
(Pappu et al., 2007; Venkataraman et al., 2008). It binds with high affinity to high density lipoproteins (HDL) but can also bind to albumin, low density lipoprotein (LDL) and very low density lipoprotein (VLDL). These lipoproteins are the main carriers of plasma S1P. Degradation of S1P is mediated by intracellular S1P phosphatases or through the irreversible cleavage of S1P by S1P lyase (Hannun and Obeid, 2008; Murata et al., 2000). S1P is thought to be produced by all cells during sphingolipid turnover and therefore, S1P lyase is vital for maintaining low concentrations of S1P in SLOs.

CCR7 also plays a critical role in egress of T cells from lymph nodes as a moderator of T cell retention (Pham et al., 2008). Removal of CCR7 from T cells showed an increase in T cell egress, and less dependence on S1P. Inhibition of lymphocyte Gαi by pertussis toxin in general showed an even greater egress from lymph nodes. This suggests that S1P promotes lymph node egress in part by overcoming retention signals by CCR7. Therefore, the simple lymphocyte-centred model of T cell egress suggests S1P enables the sensing and movement toward an S1P gradient and the overcoming of CCR7 retention signals, thereby directing cells out of lymph nodes and into the efferent lymph (Rivera et al., 2008). After exiting to the lymph and migrating through the thoracic duct, lymphocytes are released back into the blood and continue the cycle again.

1.5.4 Migration to and from other secondary lymphoid organs

Lymph nodes are not the only areas to which lymphocytes travel and encounter antigen. However, for the sake of simplicity, the process of entering a lymph node was described in the previous paragraphs. While often having a similar function, some other SLOs have slightly different processes for lymphocyte migration and homing.
1.5.4.1 Spleen

The spleen is a large, ovoid shaped organ found in the left abdominal cavity and plays an important role in the filtering of blood and detection of blood-borne antigens (Goldsby, 2003). It consists of two major regions, the red pulp and the white pulp which are divided by a diffuse marginal zone. The red pulp contains macrophages, red blood cells and few lymphocytes and is the location where old red blood cells are removed and recycled. The white pulp surrounds the branches of the splenic artery and consists mainly of T cells in the periarteriolar lymphoid sheath (PALS) and B cells in primary lymphoid follicles. The spleen is not supplied by lymphatic vessels but rather all lymphocytes are carried to the spleen via the blood through the splenic artery. Blood flows through the marginal zone and from there, lymphocytes and dendritic cells enter the white pulp. They do not have HEVs like lymph nodes but rather they pass through a layer of cells that line the marginal sinus that form a barrier between the marginal zone and the white pulp, and these cells express adhesion molecules such a MAdCAM-1, VCAM-1 and ICAM-1 (Kraal et al., 1995; Lo et al., 2003). Blood-borne antigens also pass through the splenic artery and into the marginal zone to be trapped by interdigitating dendritic cells which then migrate to the PALS to present to lymphocytes (Goldsby, 2003). Similarly to the lymph nodes, the spleen also has a FRC network which connects the marginal zone to the PALS thereby directing T and B lymphocytes using CCL21, CCL19 and chemokine (C-X-C motif) ligand 13 (CXCL13) from the blood, into the spleen, and in the direct path of APCs (Ansel et al., 2000; Forster et al., 1999; Nakano et al., 1998). After T cells have finished monitoring for foreign antigen in the spleen, they downregulate CCR7 and leave the white pulp although the exact anatomical route is still in question (Mebius and Kraal, 2005). It is believed they leave the white pulp back through the marginal zone and then re-enter the bloodstream, unlike lymph nodes that return their leukocytes to the lymph and the thoracic duct before re-entering the bloodstream.
1.5.4.2 Peyer’s patches

Peyer’s patches are organized lymphoid nodules interspersed at intervals just beneath the gut epithelium (Janeway, 2005). They are located in the lamina propria layer of the mucosa and extend further down to the submucosa of the ileum. They are composed of a specialized follicle-associated epithelium and a subepithelial dome which is abundant in dendritic cells (Fagarasan and Honjo, 2003). B cells are found in primary follicles and T cells are found in the interfollicular regions, which is also where HEVs are located. Macrophages, plasma cells and stromal cells along with the T, B and dendritic cells make up the diffuse tissues of the lamina propria. Peyer’s patches are in direct contact with the intestinal lumen and therefore are important as a first line of defense against pathogens (Goldsby, 2003). They contain specialized microfold (M) cells in their epithelial layer which are flattened epithelial cells lacking microvilli and containing a deep invagination filled with B cells, T cells and macrophages. M cells endocytose antigen from the lumen of the digestive tract and release it into the pocket containing leukocytes, thereby controlling mucosal immunity by creating tolerance as well as a quick response against more dangerous pathogens. Lymphocytes enter the Peyer’s patches through HEVs but the composition of adhesion cells is slightly different than those found in lymph nodes. It has been shown that lymphocytes with high levels of L-selectin that recognizes PNAd on HEVs prefer to migrate to peripheral lymph nodes, whereas lymphocytes that express α4β7 integrin that recognizes MAdCAM-1 prefer to migrate to Peyer’s patches, although it should be noted that L-selectin can bind to MAdCAM-1 as well (Berg et al., 1993; Butcher et al., 1999). HEVs in Peyer’s patches are L-selectin ligand low and therefore rely more on α4β7 for rolling and tethering before engaging LFA-1 and causing arrest. However, α4β7 low L-selectin naïve lymphocytes are incapable of mediating homing to MAdCAM1+ intestinal lamina propria (an extralymphoid effector site) thereby preventing naïve T lymphocytes from entering sites where they are not needed. T cells exit the Peyer’s patches and enter back into the lymph toward the thoracic duct similarly to lymph nodes (Schwab and Cyster, 2007).
1.5.5 Chemotaxis

It is critical that cells are able to travel throughout the body and to specific locations. Movement is not typically random but rather, is directed by extracellular guidance cues, termed chemotaxis. These cues, usually in the form of chemokines, interact with receptors found on the extracellular membrane of the cells that are traveling and trigger a signalling cascade that leads to cytoskeleton rearrangement, thereby polarizing the cell and causing directional migration (Berzat and Hall, 2010).

There are three types of cell movement: chemotaxis involves the movement along a gradient in which the chemoattractant is soluble, haptotaxis is the movement along a gradient where the chemoattractant is expressed or bound on a surface, or chemokinesis, which involves the random movement of cells in the presence of a chemoattractant, but not necessarily toward a higher gradient or source of the chemoattractant (Thelen and Stein, 2008). Leukocytes migrating through the blood use the process of haptotaxis because gradients are created by surface-bound chemokines. Soluble chemokines are locally produced and retained through high affinity binding to basic amino acid motifs on the chemokines to acidic glycosaminoglycans (GAGs) on blood vessel walls, preventing dispersion of the chemokine. Chemokines are often produced by cells under the vessel wall and are then presented on the luminal side of the vessel wall.

1.5.6 Chemokines

Chemokines are part of the cytokine family which stimulate surrounding cells to migrate by way of a concentration gradient (Fernandez and Lolis, 2002). This directs cells to travel to specific tissues, or within compartments of tissues. They can be secreted constitutively, such as in the example of maintaining homeostasis, or can be secreted only under inflammatory conditions to direct leukocytes to affected areas. They are secreted by many different cell types, for example stromal cells, dendritic cells, macrophages, and T cells.
As many as 50 chemokines have been identified to date (Goldsby, 2003). They generally have four invariant cysteines which form two disulphide bonds and are the key to forming their tertiary shape. They have a molecular mass of between 8 and 10 kDa, or 90-130 amino acids, and share a 20-50% homology. They are categorized into four groups based on the sequence of the first two cysteines, CC, CXC, C, and CX₃C chemokine families (Zlotnik and Yoshie, 2000). These sequences allow for a different quaternary structure for each family, although the tertiary structure of each monomer is virtually identical. The first family, CC, have two adjacent cysteine residues found near the amino terminus. While most chemokines have four cysteines, a number of CC chemokines have six cysteine residues. CXC chemokines have 17 members and can be classified further by a glutamic acid-leucine-arginine (ELR) motif found adjacent to the first cysteine residue. Chemokines that are ELR+ have been shown to promote angiogenesis whereas chemokines lacking ELR inhibit angiogenesis (Strieter et al., 1995). There are only two members in the C family, XCL1 and XCL2 and are produced by activated T cells (Kennedy et al., 1995; Yoshida et al., 1995). There is only one member in the CX₃L family, called fractalkine which is expressed predominantly in the central nervous system (Bazan et al., 1997).

Chemokine receptors on target cells are most often a seven transmembrane, G protein-coupled receptor (Goldsby, 2003). There have been 19 chemokine receptors identified to date and they are predominantly found on leukocytes. They are categorized into the same four categories based on their corresponding ligands.

Two chemokines in particular, CCL21 and CCL19, are especially important for T cell migration and homeostasis.

1.5.6.1 CCL21

CCL21, also known as 6Ckine, secondary lymphoid chemokine (SLC), Exodus-1 and thymus-derived chemotactic agent-4 is a CC chemokine containing six conserved cysteines,
two of which are located in a highly charged, carboxyl-terminal extension. It is constitutively produced by HEVs lining the lymphatic vessels of SLOs as well as lymphatic vessels of non-lymphoid tissues and binds to the receptor CCR7. CCL21 is also produced by stromal cells in T cell zones to control movement within the SLOs (Gunn et al., 1998; Kriehuber et al., 2001). In humans, CCL21 is a single 111 amino acid peptide however in mice, CCL21 is 110 amino acids long and encoded by two genes, Scya21a (CCL21-ser) and Scya21b (CCL21-leu) where they have either a serine or leucine residue at position 65, but appear to have the same bioactivity (Nakano and Gunn, 2001; Vassileva et al., 1999). They have, however, been shown in different locations. CCL21-ser is produced by stromal cells in the T cell area and by the HEVs of SLOs, whereas CCL21-leu is produced solely by the lymphatic endothelium (Gunn et al., 1998).

Paucity of lymph node T cells (plt) mice do not express CCL21-ser and CCL19 and show extreme defects in homing of naïve T cells to SLOs as well as architectural development of lymph nodes, spleen, and Peyer’s Patches (Gunn et al., 1998; Mori et al., 2001; Nakano and Gunn, 2001). CCL21 is chemotactic for thymocytes, naïve T cells, mature dendritic cells and at high concentrations, naïve B cells, but not macrophages or neutrophils (Gunn et al., 1998; Hedrick and Zlotnik, 1997; Nagira et al., 1997; Nagira et al., 1998; Tanabe et al., 1997; Warnock et al., 2000).

1.5.6.2 CCL19

CCL19, also known as Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC), Exodus-3 and macrophage inflammatory protein-3-β (MIP-3-β), is a CC chemokine containing four conserved cysteines. It is produced primarily by stromal cells in thymus and by FRCs in T cell areas of SLOs and like CCL21, also binds to the receptor CCR7 (Link et al., 2007; Yoshida et al., 1997). CCL19 presented in the HEVs is transported there from stromal cells, unlike CCL21 which is produced on site in the HEVs (Rot, 2003). CCL19-deficient mice do not show any obvious defects in the architecture and cellular composition of thymi and SLOs and show
normal rates of lymphocyte homing into lymph nodes when CCL21 is present (Link et al., 2007). CCL19 does, however, have a non-redundant role to CCL21 in which CCL19 is important for T cell homeostasis and survival having a comparable, although less important role, than IL-7. CCL19 is chemotactic for mature thymocytes, naïve T cells, mature dendritic cells but not B cells.

Both CCL21 and CCL19 have similar binding affinities to CCR7, G protein activation, calcium flux and chemotactic responses (Sullivan et al., 1999; Yoshida et al., 1998). The pathways that have been known to be stimulated upon CCL21 and CCL19 binding include the Gi – MAPK pathway shown to be important in chemotaxis, the Rho – Pyk2 pathway for migratory speed and PI3K-Akt for cell survival (Riol-Blanco et al., 2005). These signalling pathways have mostly been studied in dendritic cells and neutrophils. However, Rho, ERK, Akt and JAK signalling have been shown in T cells upon CCL21 and CCL19 binding (Bardi et al., 2003; Gollmer et al., 2009; Stein et al., 2003). There are differences in signalling between the different cell types. For example, dendritic cells stimulated with CCL21/CCL19 show only phosphorylation of ERK2 whereas neutrophils show both ERK1 and ERK2 phosphorylation (Zarbock et al., 2009).

Together, CCL21 and CCL19 regulate the homing and movement of lymphocytes and myeloid cells to spatially segregated compartments within the SLOs, thereby facilitating interactions between different cell types and optimizing cognate antigen-specific interaction. While they have similar affinities to CCR7, they can induce different signalling effects. They have different calcium mobilization and receptor desensitization efficacies as CCL19 stimulates CCR7 phosphorylation and internalization, whereas CCL21 does not (Kohout et al., 2004). This implies that CCL19 signalling is short lived as compared to responses caused by CCL21 that does not have receptor desensitization after binding.
1.6 PSGL-1

Migration of cells has been shown to be heavily dependent on adhesion molecules. They play important roles both in mediating slowing down and arrest of cells so that they can enter appropriate tissues, as well as signalling cells to upregulate or activate other adhesion molecules. P-selectin glycoprotein ligand -1 (PSGL-1) is one such protein that is of great interest as its role in the lymphocyte migration is continually being expanded.

PSGL-1 is a well characterized protein known for its role as a selectin ligand capable of binding all three selectins with a low affinity. L-selectin is found on leukocytes, E-selectin on endothelial cells and P-selectin on both platelets and endothelial cells. PSGL-1 plays a role in the rolling and tethering of leukocytes in the blood vessels of inflamed tissues, causing the leukocytes to slow down, interact with chemokines and eventually arrest and migrate into the tissues where they can carry out their effector cell functions.

1.6.1 Structure and expression

Human PSGL-1 was first discovered by Western blot in 1992 by the McEver lab using P-selectin as a probe on human neutrophil and HL-60 cell membranes, hence its name (Moore et al., 1992). It is a type I membrane glycoprotein found on the surface of most hematopoietic cells with the exception of red blood cells, megakaryocytes and debatably platelets. PSGL-1 is, however, found in varying amounts and with various post-translational modification states depending on cell type. B cells express lower levels than other blood leukocytes, and naïve T cells lack branched core 2 structures necessary for selectin binding. It can also be expressed on vascular endothelial cells but has only been seen under chronic inflammatory conditions (Rivera-Nieves et al., 2006).
PSGL-1 is a heavily glycosylated homodimeric mucin containing two 120kDa subunits held together by a disulfide bond mediated by a single cysteine found near the transmembrane domain (Figure 1.3). Its extended regions include 14-16 decameric repeats that are rich in serine, threonine and proline. These regions have potential binding sites for two to three N-glycans as well as numerous O-glycans, critical to its function as a selectin ligand (Lozano et al., 2001). It has three N-terminal tyrosines that can be sulphated, necessary for ligand binding (Sako et al., 1993). The cytoplasmic domain has no consensus sequences for phosphorylation. However it does support signalling through the binding of the ezrin, radixin, and moesin (ERM) family. The ERM family are found just below the plasma membrane and are believed to be involved in actin filament/plasma membrane association (Tsukita et al., 1994).

Murine PSGL-1 has structural and functional homology to human PSGL-1. However, its N-terminus is poorly conserved. It has only ten decameric repeats in comparison to the 14-16 of human PSGL-1. It also contains many serines, threonines and prolines, as well as the single cysteine near the transmembrane domain, all features of a mucin-like protein. The transmembrane and cytoplasmic domains on the other hand share roughly 60% conservation (Baisse et al., 2007). It has two N-terminal tyrosines that are sulphated and required for ligand binding as opposed to three in humans.
1.6.2 Posttranslational modifications

The function of PSGL-1 as a selectin ligand is dependent on its posttranslational modifications which are closely regulated by various glycosyltransferases. The carbohydrate selectin ligand is found at the N-terminal of PSGL-1, branched from an O-glycan at threonine 17 in mice, or threonine 16 in humans. The glycosylation pattern is termed sialyl Lewis x (sLe\(^x\)) and consists of an N-acetylgalactosamine bound to a fucose and a galactose bound to a sialic acid (Figure 1.4). Along with tyrosine sulfations at locations 13 and 15 in mice, or 6, 9 and 11 in

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**Figure 1.4: Posttranslational modifications of O-glycans on PSGL-1.** Order in which selectin ligand motifs are built on serine or threonine residues:
- **ppGalNAcT** – polypeptide N-acetylgalactosamine transferase
- **C1β1,3GalT** – Core 1 β 1,3 - galactosyltransferase
- **C2β1,6GnT-1** – Core 2 β 1, 6 – N-acetylgalactosaminyltransferase
- **β 1,4GalT** – β 1, 4 - galactosyltransferase
- **α2,3ST3GalT1** – α 2, 3 – sialytransferase 1
- **α 1,3FuT7** – α1,3 fucosyltransferase 7

Posttranslational modifications, and thereby ligands, are controlled in part by the order in which carbohydrates are added. When certain transferases are absent (namely C2GnT-1 and FuT7), the sLe\(^x\) motif cannot form and selectins cannot bind.
humans, it is only this region that selectins are able to bind. E-selectin is the exception in that it can bind the N-terminal region as well as O-glycan sites along the decameric repeats of PSGL-1 (Goetz et al., 1997). The sLe\(^x\) selectin ligand is expressed on all myeloid cells, but not on naïve lymphocytes (Moore et al., 1995). Myeloid cells constitutively express selectin ligand because they constitutively express the glycosyltransferases needed for their formation. However, naïve lymphocytes do not express glycosyltransferases critical for selectin ligand formation. After activation of lymphocytes, upregulation of Core 2 β1,6-N-acetylglucosaminyltransferase (C2GnT)-1 and fucosyltransferase (FucT) VII modulates selectin ligand formation on PSGL-1 (Ley and Kansas, 2004). There are various factors than can regulate selectin ligand expression including substrate availability and competition for substrate. Glycosyltransferases are located at specific regions in the Golgi and proteins encounter each one sequentially. Some glycosyltransferases, such as ST3Gal1 found in the trans-Golgi and C2GnT-1 found in the cis-Golgi can both modify core 1 structures and therefore their competition will determine which substrate a protein will be capable of binding to. External factors such as cytokines may mediate the upregulation of glycosyltransferases. However it has not yet been fully elucidated. It was suggested the IL-2 and IL-12 are important in selectin ligand formation \textit{in vitro} yet these cytokines appear to be dispensable \textit{in vivo} (Carlow et al., 2005). There are also external factors that mediate the suppression of selectin ligand formation, such as retinoic acid (Iwata et al., 2004).

1.6.3 Binding partners

The main function of PSGL-1 is as a selectin ligand. All three selectins are structurally similar containing an N-terminal lectin-like domain, an epidermal growth factor-like domain, consensus repeats, a single-transmembrane domain and a short cytoplasmic tail (Kelly et al., 2007). P-selectin has nine consensus repeats, whereas E-selectin only has six, which cause the molecules to extend beyond the negatively charged glycocalyx of the endothelium, allowing
leukocyte-endothelial cell interactions (Patel et al., 1995). L-selectin only has two consensus repeats. However it is localized on protruding microvilli of the leukocyte thereby also extending from the cell to encourage optimal leukocyte-endothelial cell interactions (Erlandsen et al., 1993).

1.6.3.1 P-selectin

Gene deletion and antibody blocking studies show that PSGL-1 is the dominant ligand for P-selectin, mediating leukocyte rolling on inflamed endothelial cells \textit{in vivo} (Borges et al., 1997; Hirata et al., 2000; Norman et al., 1995; Xia et al., 2002). Very few PSGL-1\textsuperscript{null} leukocytes roll on P-selectin on activated venules, but those that do, do so very irregularly and more rapidly than WT leukocytes. P-selectin is present in storage granules, called Weibel-Palade bodies, in the endothelium of blood vessel walls and the α-granules in platelets under homeostatic conditions. Endothelial cells are activated under inflammatory conditions by factors such as thrombin, histamine or complement components, causing the Weibel-Palade bodies to fuse with the plasma membrane. This brings P-selectin to the surface of the cell within minutes of activation, providing a ligand for PSGL-1 on leukocytes. P-selectin has been shown to be able to be internalized and recycled in new Weibel-Palade bodies thereby allowing it to be a readily available molecule under time pressured inflammatory conditions (Subramaniam et al., 1993). TNF-α, IL1-β and lipopolysaccharide (LPS) has been shown to augment mRNA transcription of P-selectin in murine endothelial cells and IL-4 and IL-13 have been shown to upregulate transcription in human endothelial cells (Varki, 2009; Woltmann et al., 2000; Yao et al., 1996). Activated platelets are able to adhere to neutrophils, monocytes, NK cells and activated T cells through P-selectin and PSGL-1. This augments the recruitment of leukocytes to the site of vascular injury. P-selectin is able to bind heparin/heparan sulphate and some glycoproteins with specific glycosylation patterns, but its major ligand is PSGL-1. P-selectin is only able to bind PSGL-1 if the latter is properly glycosylated with the sLe\textsuperscript{X} motif on its N-terminus as well as
proper tyrosine sulfation (Figure 1.5). sLe\(^x\) is constitutively expressed on PSGL-1 found on granulocytes and monocytes, but is only present on lymphocytes when activated and is absent on naïve and resting lymphocytes.

**Figure 1.5: P-selectin binding to PSGL-1.** P-selectin (and L-selectin) binds to the N-terminus of PSGL-1 where the sLe\(^x\) is present at its specialized location on either arm of the homodimer. E-selectin binds this region as well, but is also capable of binding to the numerous O-glycans that decorate the stem of PSGL-1. This configuration allows for catch bonds.

1.6.3.2 **E-selectin**

E-selectin, in contrast to P-selectin, is not constitutively expressed in endothelial cells but rather is produced *de novo* when activated by TNF-α, IL1-β and LPS, mediated by the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Varki, 2009). Therefore, E-selectin only appears on the surface of endothelial cells within hours of activation and decreases within 24 hours (Kansas and Pavalko, 1996). E-selectin is also degraded after being internalized, not recycled as is the case for P-selectin (Subramaniam et al., 1993). E-selectin has a number of ligands including CD44 and E-selectin ligand-1 (ESL-1) in addition to PSGL-1. On neutrophils it has been suggested that each has its own function: PSGL-1 initiates tethering, ESL-1 supports tethering and slow rolling and CD44 facilitates arrest, partly through signalling (Hidalgo et al., 2007). E-selectin binding to PSGL-1 is less stringent than P-selectin binding to PSGL-1. It is tyrosine sulfation independent and can bind multiple O-
glycan sites whereas P-selectin (and L-selectin) are limited to threonine 17 (16 in humans) at the N-terminus of PSGL-1 (Goetz et al., 1997; Martinez et al., 2005; Tenno et al., 2007).

1.6.3.3 L-selectin

L-selectin differs from the other two selectins in that it is found on all classes of leukocytes and plays a critical role in the homing of leukocytes to SLOs under homeostatic conditions as opposed to inflammatory conditions like its other two family members (Tedder et al., 1990). Its major ligands are GlyCAM-1, CD34, MAdCAM-1 and PSGL-1 (Berg et al., 1993; Mebius et al., 1993; Puri et al., 1995; Tu et al., 1996). Its traditional function is as a homing adhesion molecule with GlyCAM-1 which is found on the HEVs of lymph nodes, allowing the leukocyte to slow down before coming to an arrest with the aid of other adhesion molecules like LFA-1 and ICAMs. (See figure 1.2 for rolling and tethering cascade in HEVs) It has also been shown to be important in inflammatory settings as it mediates leukocyte-leukocyte interactions (Winn et al., 1998). PSGL-1 and L-selectin on two cells interact with each other as a tethering bond, increasing migration of leukocytes to inflamed tissues. L-selectin optimally binds 6-sulfo-sLe\(^x\), which is sulphated on the C-6 of the GlcNAc. It is found on L-selectin binding partners in the HEVs, but not on PSGL-1 (Rosen, 2004). Studies show that L-selectin prefers ligands with 6-sulfo sLe\(^x\) rather over sLe\(^x\) with tyrosine sulfation whereas P-selectin prefers the opposite (Kanamori et al., 2002). L-selectin also has a 10-25 fold lower affinity for binding PSGL-1 than P-selectin (Cheng et al., 2008; Leppanen et al., 2000; Leppanen et al., 2003).

1.6.3.4 Selectin catch bonds

The binding of selectins with PSGL-1 is unique in that it is a low affinity bond, constantly binding then releasing causing the rolling and tethering action of the leukocyte along the blood vessel wall. It is a catch bond, a noncovalent bond in which the bond lifetime increases when an increased tensile force is applied. For the case of leukocytes, cells will roll when in the presence of a low shear but will firmly adhere in the presence of a high shear, or, if shear threshold has
not been reached, they may not bind at all (Thomas, 2008). Normally, bond lifetimes decrease with augmented applied force which makes this an interesting phenomenon. There are two proposed models of PSGL-1 flow-enhanced adhesion, the sliding/re-binding model and the induced high affinity model (Carlow et al., 2009).

The sliding/re-binding model suggests that the P-selectin is in a kinked configuration while in a resting state between its proximal EGF domain and its distal lectin-binding ectodomains. The tension associated with the tethering of PSGL-1 causes P-selectin to straighten its configuration. The flow enhances tethering rates and the extended P-selectin structure provides better access to pre-existing binding sites. The opening of the protein would cause better alignment with the direction of the sheer force, thereby promoting sliding of the ligand, causing dissociation. However, new interactions would quickly form to replace those that were disrupted or for the original interactions to reform (Zhu et al., 2008).

The induced high affinity model also suggests that P-selectin straightens its configuration but that it is this extension that induces allosteric changes that leads to enhanced binding affinity between PSGL-1 and P-selectin (Konstantopoulos et al., 2003). The epidermal growth factor-like domains and the sLe\(^\text{X}\) that are critical to this mechanical stability (Alon and Rosen, 2007). There are ligands that are recognized by soluble selectins, such as heparin sulphate and chondroitin sulphate, but lack these two components and therefore cannot support stable adhesive interactions under shear flow.

1.6.3.5 Other binding partners

PSGL-1 also binds various other molecules including von Willebrand factor (VWF) and versican. VWF is a large multimeric glycoprotein that can be found in the Weibel-Palade bodies of endothelium along with P-selectin and other components such as chemokines and growth factors (Rondaij et al., 2006). When endothelium is damaged, or inflammatory cytokines are
present, the contents of Weibel-Palade bodies are released to the surface of the cell and VWF unfurls to ultralong filaments that capture platelets causing the formation of a hemostatic plug (Michaux et al., 2006). Platelets form an adherent layer on the endothelium with its PSGL-1 interacting with either P-selectin, or VWF, depending on the force of the shear. Venules with higher shear will form PSGL-1-P-selectin interactions, while veins with lower shear will form PSGL-1-VWF interactions (Andre et al., 2000). It has also been shown that the N-terminal tyrosine sulphate-rich regions of PSGL-1 on granulocytes and monocytes are able to bind to VWF allowing for an additional binding partner in the leukocyte rolling and adhesion (Pendu et al., 2006). Versican is a major chondroitin sulphate proteoglycan and can be found on the large artery endothelia and vascular smooth muscle. It has been shown that the extracellular domain of PSGL-1 can bind versican in vitro causing leukocyte aggregation. The physiological relevance, however, is still unknown (Zheng et al., 2004).

1.6.4 Function

1.6.4.1 Cell migration under inflammatory conditions

As previously described, through the interactions of P-selectin and E-selectin on inflamed venules, PSGL-1 plays an important role for leukocyte migration during an immune response. In addition, PSGL-1 is able to bind L-selectin on neighbouring leukocytes to increase T cell migration to areas of inflammation. However, PSGL-1 has also been implicated in various other roles of leukocyte migration under alternate conditions.

Hematopoietic stem cells (HSCs) are decorated with PSGL-1 and can travel through the body into the bone marrow under extenuating circumstances, such as irradiation. P-selectin and E-selectin can be found in "hotspots" in the sinusoids and collecting venules of the bone marrow network, and blocking these selectins severely reduces the ability of human adult HSCs to roll and home to the bone marrow by about 90% (Hidalgo et al., 2002). In mice, HSC homing seems
to be less dependent on PSGL-1 as rolling is only reduced by 35% when Ab blockade against PSGL-1 is used (Katayama et al., 2003). Further reduction by approximately 90% was seen when E-selectin and α4 integrin were inactivated. E-selectin or P-selectin-deficient mice showed impaired HSC homing and additional Ab blockade against VCAM-1, binding partner of α4 integrin, showed a greater impairment (Frenette et al., 1998). This suggests that P-selectin, E-selectin, and VCAM-1 on bone marrow vasculature and PSGL-1 and α4 on HSCs are the important adhesion molecules that mediate HSC rolling and homing. Interestingly, neonatal human HSCs derived from cord blood show reduced osteotropism compared to adult HSCs because 30% of neonatal HSCs have non-functional P-selectin ligand (Hidalgo et al., 2002). These cells have reduced levels of FucT VII. However when they were treated with recombinant FucT, the neonatal HSCs upregulated functional selectin ligand and showed increase interaction with bone marrow vasculature, but were still unable to home into the bone marrow (Hidalgo and Frenette, 2005).

1.6.4.2 Cell migration under non-inflammatory conditions

More recently, PSGL-1 has been implicated in non-inflammatory settings. Plasma B cells and central memory CD8+ T cells reside in the bone marrow for substantial periods of time. B cells upregulate PSGL-1 during maturation from naïve B cells to plasma B cells and preferentially bind E-selectin (Zhan et al., 2003). This may suggest a role for PSGL-1 in the homing of plasma B cells to bone marrow as they spend long periods of time there producing antibodies (Cyster, 2003). Central memory CD8+ T cells also spend time in the bone marrow and rely predominantly on selectins for rolling, and VCAM-1/α4β1 for firm adhesion (Mazo et al., 2005).

PSGL-1 also plays an important role in the homing of T cell progenitors (TCPs) to the thymus (Rossi et al., 2005). The thymus does not contain any self renewing cells and therefore relies upon the importation of TCPs from the bone marrow through the blood. TCPs express
functional selectin ligand and antibody inhibition against selectins demonstrate that P-selectin is necessary for TCPs entry into thymus, but not E-selectin or L-selectin. TCPs lacking PSGL-1 or C2GnT-1 (needed for selectin ligand formation), are unable to seed the thymus as effectively as WT TCPs. P-selectin\textsuperscript{null} recipients have reduced numbers of TCPs within the thymus (Gossens et al., 2009). Interestingly, once thymi are “full” they downregulate P-selectin on their endothelium to prevent TCP entry, and upregulate it again when the thymus is ready to accept more progenitors.

1.6.4.3 Signalling

Another function of PSGL-1, other than as an adhesion molecule in the rolling and tethering cascade, is as a signalling molecule. Most signalling studies involving PSGL-1 have been done with neutrophils or cell lines. Signalling has been shown along at least three different pathways. First, PSGL-1, upon stimulation with either P-selectin or PSGL-1 antibodies, interacts with ezrin and moesin, which are part of the ERM family of proteins known to act as linkers between the actin cytoskeleton and plasma membrane proteins as well as signal transducers involving cytoskeletal remodelling (Ivetic and Ridley, 2004). It was hypothesized that ezrin and moesin may be responsible for positioning PSGL-1 at the uropod of migrating cells (Alonso-Lebrero et al., 2000). ERM proteins also contain an immunoreceptor tyrosine-like activation motif (ITAM)-like motif in their N-terminal region. When this domain is phosphorylated on moesin, it is capable of recruiting and activating spleen tyrosine kinase (Syk) (Urzainqui et al., 2002). Syk then activates c-Fos which is important for cell cycle regulation, apoptosis, cell growth and cell differentiation. Syk is expressed in a wide range of hematopoietic tissues. It also has a structural and functional homologue, ZAP-70 that is found only in T cells and NK cells (Chu et al., 1998). Both these signalling molecules are also known to be downstream of the TCR receptor.
Nef-associated factor 1 (Naf1) is constitutively bound to the cytoplasmic tail of PSGL-1 at a binding site that overlaps with the ERM binding site (Zarbock et al., 2009). When PSGL-1 binds P-selectin, Src kinases phosphorylate Naf1 which in turn recruits PI3K for the activation of integrins (Wang et al., 2007). Naf1 has also been shown to bind to extracellular-signal-regulated kinase (ERK) 2 thereby preventing it from entering the nucleus and down regulating nuclear signalling (Zhang et al., 2002). Both of these pathways are also stimulated by CCR7.

Lastly, PSGL-1 binding to P-selectin activates the Src-family kinases Lyn and Hck while E-selectin binding activates Fgr and Hck (Piccardoni et al., 2001). Fgr has been shown to be required for LFA-1 activation and slow rolling (Zarbock et al., 2008). However, in dendritic cells lacking Fgr, CCL21 and CCL19 mount greater chemotactic responses suggesting that Fgr plays a role in a negative feedback loop of CCR7 signalling, whereas the same effect could be shown for double knockouts of Fgr and Hck in neutrophils.

PSGL-1 signalling pathways can also be linked to the CCR7 signalling pathways through numerous signalling molecules (Figure 1.6). CCR7 signalling in dendritic cells is known to occur through three main pathways, the MAPK, Rho, and PI3K pathways which have all been shown to be influenced by signalling molecules downstream of PSGL-1 (Riol-Blanco et al., 2005). The PI3K/Akt signalling plays a role in the survival and viability of cells. The ERK1/2/JNK signalling pathway is necessary for chemotaxis and the Rho/Pyk2 signaling pathway is involved in the migratory speed of cells.
Figure 1.6: PSGL-1 and CCR7 signalling upon ligand binding. CCL21 and CCL19 bind their receptor CCR7, a G protein coupled receptor, which signals through the PI3K, ERK1/2 or Rho pathways for various functions. PSGL-1 signals through ERM and Naf1 upon binding with P-selectin or E-selectin in inflammatory settings. Many signalling molecules from CCR7 and PSGL-1 have been shown to interact under certain circumstances.
1.7 Thesis objectives

While investigating the role of PSGL-1 in thymic progenitor homing in parabiotic mouse models, it was noted that PSGL-1-deficient T cells were approximately 50% reduced in lymph nodes compared to WT T cells (Rossi et al., 2005). Furthermore, PSGL-1\textsuperscript{null} mice were found to have a reduced thymic output and decreased numbers of CD4+ and CD8+ T cells in the peripheral blood (Gossens et al., 2009). These results could not be explained by the current understanding of PSGL-1 as a selectin ligand that functions in homing and migration of activated T cells but not resting T cells. Therefore, we hypothesized that PSGL-1 may play a role in steady state homeostasis of T cells. The overall objective of this study is to determine the role of PSGL-1 in non-inflammatory settings, both in terms of migration of T cells as well as T cell homeostasis. Specifically, we will identify the role of PSGL-1 in T cell homing to and from SLOs as well as the influence of PSGL-1 on T cell homeostatic mechanisms.
CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

Mice used were between the ages of 5-10 weeks. They were bred and housed in the specific pathogen free animal unit at the Biomedical Research Centre. LCMV infected mice were housed at Wesbrook Animal Unit. All mice are on a C57BL/6 background, or backcrossed at least ten generations on a C57BL/6 background. Double knockouts and heterozygotes were produced in house through matings of single knockout or homozygote allele mice.

Table 2.1: Mice used in experiments

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (CD45.2)</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>WT (CD45.1)</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>WT (CD45.1/CD45.2)</td>
<td>In house matings</td>
</tr>
<tr>
<td>PSGL-1null (Thy1.2)</td>
<td>Jackson Laboratories (Furie genotype)</td>
</tr>
<tr>
<td>PSGL-1null (Thy1.1)</td>
<td>In house matings</td>
</tr>
<tr>
<td>PSGL-1null (GFP)</td>
<td>In house matings</td>
</tr>
<tr>
<td>P-selectinnull</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>E-selectinnull</td>
<td>Dr. D. Bullard (University of Alabama at Birmingham)</td>
</tr>
<tr>
<td>L-selectinnull</td>
<td>Dr. S. Rosen (University of California at San Francisco)</td>
</tr>
<tr>
<td>C2GnT-1null</td>
<td>Dr. J. Marth (University of California at San Diego)</td>
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<tr>
<td>PSGL-1null/C2GnT-1null</td>
<td>In house matings</td>
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<td>CD43null</td>
<td>Dr. A. Sperling (University of Chicago)</td>
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<td>OT-I</td>
<td>Dr. F.R. Carbone (University of Melbourne)</td>
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<td>OT-I/PSGL-1null</td>
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<td>HYgnull</td>
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<td>HYg/PSGL-1null</td>
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<td>IL-7Rnull</td>
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<td>IL-7Rnull/P-selectinnull</td>
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</tr>
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</table>

2.2 Tissue extraction

Mice were sacrificed using CO₂. Superficial cervical, brachial, inguinal, mesenteric and axillary lymph nodes, spleen and Peyer’s patches were manually removed using forceps. Lymph nodes, spleens and Peyer’s patches were dissociated into single-cell suspensions with a stainless steel sieve in complete RPMI (Gibco) (containing 10% (vol/vol) FBS, 2 mM L-
glutamine, 50 μM 2-mercaptoethanol, penicillin and streptomycin) and kept at 4°C. For cases where lymph node counts were taken, lymph nodes were cut into smaller fragments and dissolved with 0.5 mg/mL collagenase/dispace (Roche) for 2 hours at 37°C in HBSS on a turning rack with 10 μg/mL DNase (Roche). Peripheral blood was taken either by perfusion with PBS + 4% FBS + 2.5 μM EDTA, or, for exact measurements, by cardiac puncture. Red blood cell lysis was performed on peripheral blood and spleen.

2.3 Congenic marker system

The congenic markers CD45.1/CD45.2 and Thy1.1/Thy1.2 (CD90.1/CD90.2) are a system in which to distinguish donor cells from hosts. CD45 is a protein tyrosine phosphatase known as the leukocyte common antigen and is expressed on all bone marrow derived cells with the exception of erythrocytes and erythroblasts and exists in multiple isoforms (CD45.2 and CD45.1) which can be distinguished by specific antibodies. Thy1 is a heavily glycosylated GPI anchored conserved cell surface protein found on thymocytes and peripheral T cells. Thy1.1 has an arginine at position 108 whereas Thy1.2 has a glutamine allowing for antibody distinction.

2.4 Flow cytometry

Cell suspensions were stained with indicated antibodies (see table 2.2) in FACS buffer (PBS + 2% FBS (vol/vol) + 2 μM EDTA) for 20 minutes at 4°C (on ice) in the dark. They were washed 2x in FACS buffer. Cells stained with biotinylated antibodies were then stained with streptavidin antibodies for 10 minutes at 4°C (on ice) in the dark. CCR7 mAb is the exception in that it was stained at 37°C as recommended. Where mentioned, propidium iodide (PI) was added to final sample volume at 250 ng/mL. Counting beads (Invitrogen) were added to some samples to determine absolute cell numbers. Counting beads were used as per the instructions. In each sample, a diluted portion of beads were added to each FACS sample just before analysis on the flow cytometer. Beads were gated in software based upon size. Cell samples
were then enumerated based upon the standardization of reference beads using the equation:

\[
\text{(number of cells events/number of beads events) x (assigned bead count/volume of sample)}
\]

Samples were analyzed on a FACScan, FACSCalibur or lsrII flow cytometer (BD Bioscience).

Data was analyzed using Flow Jo (Tree Star software).

Table 2.2 Antibodies used in experiments

<table>
<thead>
<tr>
<th>Target</th>
<th>Species</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Source</th>
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2.5 In vivo homing experiments

Single cell suspension lymph node cells were labeled either with 0.2 µM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, referred to as CFSE, Molecular Probes, Invitrogen) for 5 minutes at 25°C in Hanks balanced salt solution (HBSS - from Gibco) or with 8 µM cell tracker orange (CTO; Molecular Probes, Invitrogen) for 8 minutes at 37°C in
HBSS. Immediately after staining, cells were washed once with complete RPMI then once with HBSS. A 1:1 ratio of WT and PSGL-1\textsuperscript{null} cells was determined by the cell count on the hemocytometer then verified by flow cytometry prior to injection. Cells were combined based on flow cytometry data. A final cell count was taken using counting beads. Samples were resuspended in HBSS at 25 x 10\textsuperscript{6} cells and 200 µl were injected intravenously into the tail vein of either WT, P-selectin-deficient or E-selectin-deficient recipients. Mice were sacrificed after 60 minutes in one hour homing experiments, or after specified number of days for peripheral blood disappearance experiments, before sacrificed in order of injection. Peripheral blood, brachial, inguinal, mesenteric and axillary lymph nodes, spleen and Peyer’s patches were collected and analyzed by flow cytometry.

### 2.6 T cell egress

HY and HY/PSGL-1\textsuperscript{null} thymi were mashed in a stainless steel sieve and were dissociated into a single cell suspension. Then, CD8+ single positive T cells were isolated using the EasySep system against CD4+ cells as per the instructions (Stem Cell). Isolated cells were stained with either 13 µM CFSE or 2 µM CFSE for 5 minutes in HBSS at 25°C, washed and resuspended in HBSS. They were injected into WT mice. 24 hours later, one set of mice was sacrificed, while remaining mice were injected with 150 µg each anti-α4 and anti-αL integrin antibodies (clones PS/2 and TIB217 respectively, produced in house) \textit{i.p.} per mouse. 48 hours later, one set of mice was sacrificed while the remaining mice were injected again with integrin antibodies. 72 hours later, the final set of mice was sacrificed. Lymph nodes were harvested from mice, dissociated, labelled with antibodies and analyzed on the FACSCalibur.

### 2.7 Chemokines

CCL21, CCL19, CXCL12, CXCL9 and CXCL11 were chemically synthesized ‘in-house’ with tertiary butyloxycarbonyl solid-phase chemistry. Chemokines were purified by high-
performance liquid chromatography, and mass was confirmed by electrospray mass spectrometry. Biotinylated CCL21 was produced by the coupling of biotinamidohexanoic acid N-hydroxysuccinimide ester (Sigma) to the N terminus of CCL21 before the ‘deprotection’ and refolding steps (Clark-Lewis et al., 2003).

2.8 Competitive transwell chemotaxis assay

Chemotaxis buffer (600 μL; RPMI medium, 100 mM HEPES, pH 7.2, and 0.5% (wt/vol) BSA) containing chemokine was added to the lower chamber of a transwell plate (Costar), followed by prewarming for a minimum of 1 hour at 37°C. GFP- or dye-labeled cells were transferred to chemotaxis buffer prewarmed to 37°C and were resuspended at a density of 1x10^7 viable cells per mL. In Syk and ZAP-70 inhibition experiments using piceatannol (Cayman Chemical), indicated amounts were added to cells. Prepared mixed cells (100 μL) were added to the upper transwell chamber, followed by incubation for 2–3 hours at 37°C. Migrated cells were quantified by flow cytometry and counting beads. Flow cytometry data were analyzed with FlowJo and Excel software. Absolute cell counts were normalized for differences in starting numbers and were adjusted relative to ‘no-chemokine’ controls.

2.9 Chemokine binding assays

Biotin-CCL21 (0.67 nM) and 1.1 μg fluorescein isothiocyanate (FITC)–streptavidin (BD Pharmingen) were mixed and were preincubated for 30 minutes in 30 μL chemotaxis buffer plus 2% (wt/vol) BSA, then were mixed with 1x10^6 lymphocytes in 70 μL chemotaxis buffer plus 0.5% (wt/vol) BSA, followed by incubation for 2 hours at 37 °C. Cells were then washed once with 10% (vol/vol) FBS in PBS, and were stained for lymphocyte markers CD4, CD8 or B220. After 20 minutes of incubation, cells were washed twice in 10% (vol/vol) FBS in PBS and were analyzed with a FACSCalibur.
2.10 In vivo homeostasis and proliferation

Lymph nodes were removed from each genotype of mouse and placed in a single tube and then dissociated into a single cell suspension in complete RPMI. They were washed 2x then stained with 2 μM CFSE in HBSS for 5 minutes at 25°C. Cells were washed in complete RPMI 2x then resuspended in HBSS at 25 x 10^6 cells/mL. 200 μl of cells were injected into each mouse i.v. in their tail vein. Input cells were measured by FACS immediately. WT mice in long term proliferation assays were sacrificed after 4-5 weeks. IL-7Rnull mice were sacrificed after 4 days. Irradiated WT mice were sacrificed after 7 days. Lymph nodes and/or spleens collected as previously noted, and analyzed by flow cytometry. Donor cells were recovered using the congenic system using antibodies against Thy1.1, Thy1.2 and/ or CD45.1.

2.11 DOP

DOP (4′ deoxypyridoxine, Sigma) in vivo homing experiments were performed exactly as in vivo homing experiments described above, with the addition of DOP, added at a concentration of 100 mg/L along with 10 g/L glucose to the drinking water of injected mice upon time of injection (Schwab et al., 2005). Water was refreshed approximately every two weeks. After 5 weeks, mice were sacrificed and lymph nodes were collected, as well as peripheral blood to confirm effectiveness of DOP treatment.

2.12 Bone marrow reconstitution

Bone marrow was extracted by flushing femurs and tibias with complete RPMI and manual dissociation by pipetting. Cells were then subjected to red blood cell lysis and counted on a hemocytometer. 2x10^6 cells were resuspended in HBSS and injected i.v. into recipients. Lymph nodes, thymi, peripheral blood and spleens from recipient mice were harvested 4-5 weeks later and analyzed for chimerism based on congenic markers.
2.13 *In vitro* homeostasis and proliferation

96 well plates were prewarmed with complete RPMI and the amount of indicated cytokine. Lymph nodes were removed from each genotype of mouse and placed in a single tube and then dissociated into a single cell suspension in complete RPMI. They were washed twice then stained with 2 μM CFSE in HBSS for 5 minutes at 25°C. Cells were washed in complete RPMI twice again, and then counted on a hemocytometer. Cells were added to the cytokine enriched media in the 96 well plate to a final concentration of 1 x 10^6 cells per well and kept at 37°C with 0.5% CO₂ in the dark. Input cells were measured by FACS immediately. For Syk inhibition assays, piceatannol was added to wells at the concentration indicated. In certain experiments, piceatannol was readded to cells every 24 hours to ensure the inhibitor remained active. For naïve and memory T cell *in vitro* experiments, cells were sorted by the UBC Flow Cytometry Facility on a FACS Aria (BD Biosciences) for CD8β+ and CD44^{high} and CD44^{low} cells. After sorting, cells were counted, combined and labelled with CFSE and added to cytokine enriched 96 well plate at a concentration of 3x10^5 cells/mL. After indicated number of days, cells were washed with FACS buffer, stained with indicated antibodies, washed 2x with FACS buffer again and then run on the IsrII with counting beads and PI stain. All analyses are of viable, PI negative cells.

2.14 T cell activation

Lymph nodes were removed from each genotype of mouse and placed in a single tube and then dissociated into a single cell suspension in complete RPMI. They were washed 2x then stained with 2 μM CFSE in HBSS for 5 minutes at 25°C. Cells were washed in complete RPMI 2x then plated in a 6 well plate with 4 μg/mL concanavalin A (ConA), complete RPMI and 2% (vol/vol) conditioned IL-2 media. They were kept at 37°C with 0.5% CO₂ in the dark. Cells were harvested after 2 days and washed before staining with antibodies and FACS analysis.
2.15 LCMV mouse model

7 week old sex matched mice were i.p. injected with $1 \times 10^5$ pfu LCMV-Armstrong (kindly provided by Marc Horwitz). Spleens were harvested on either Day 8 or Day 40 post-infection, dissociated into single-cell suspensions with a stainless steel sieve and treated with red blood cells lysis buffer. For tetramer staining, splenocytes were initially stained with PE-labeled gp33 tetramer or PE-labeled np396 tetramer in FACS buffer (PBS with 2% FBS (vol/vol) and 2.5 µM EDTA) for 40 minutes at 4°C in the dark. They were then washed FACS buffer and subsequently stained with CD8-APCCy7 and CD4-PECy7 for 20 minutes at 4°C in the dark. Samples stained by gp33 tetramer were immediately run on the LsrII. Samples stained by np396 were first fixed with a 1% (wt/vol) paraformaldehyde/PBS solution for 15 minutes, then run on the LsrII. Counting beads were added to all samples run on the FACS. Tetramers were kindly provided by John Priatel.

2.16 Statistics

Data are represented as mean values and error bars are depicted using standard deviation. Statistical significance was measured using an unpaired, two-tailed Student’s t-test with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Statistics were calculated using Microsoft Excel software.
CHAPTER 3: RESULTS

3.1 Introduction and rationale

The movement of T cells through the body is a complex and highly regulated process. T cells use a multitude of proteins to mediate their movement dependent on their activation state. L-selectin on the cell surface of naïve T cells mediates the process of exiting the blood stream and entering the secondary lymphoid tissues. Within these tissues, T cells monitor the antigen presenting cells (APCs) for evidence of infection before exiting the lymph nodes via the lymph and re-enter the blood stream. They continue this cycle until they come across an APC that expresses their cognate antigen, stimulating them to activate and proliferate. Part of the activation process includes shedding L-selectin, presumably to help prevent re-entry into lymph nodes. Also, transferases such as C2GnT-1 are upregulated leading to the expression of the sLe⁺ motif on PSGL-1, modulating binding to P-selectin and E-selectin found on the blood vessels near the region of inflammation. The role of selectins and PSGL-1 are critical in this process.

Much of the knowledge of PSGL-1 is based on the activated, core 2 glycosylated state of the protein, the selectin ligand, which is the steady state on most leukocytes, such as neutrophils, dendritic cells and macrophages. Thymic progenitors traveling from the bone marrow to the thymus also use core 2 glycosylated PSGL-1 to enter the thymus, as PSGL-1 has also been shown to bind P-selectin found on endothelial cells of the thymus (Rossi et al., 2005).

Lymphocytes are unique in that they do not express functional selectin ligand in resting state. This was first assumed to be for prevention of entry of naïve lymphocytes into areas of

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inflammation. This study focuses on the unique and yet-undetermined role of PSGL-1 as a non-selectin ligand both in the movement of naïve T cells through the body as well as a factor in maintaining the balance of T cell numbers through homeostatic proliferation and survival. It will be shown that PSGL-1 on naïve T cells has a much more extensive role than originally thought. While looking at T cell egress from the thymus to the periphery, it was first noted that there were discrepancies in the T cell repertoire in the peripheral blood of the PSGL-1null mouse, prompting us to look further into the homeostatic lymphocyte profiles of these mice (Gossens et al., 2009).

3.2 Results

3.2.1 PSGL-1null mice show subtle defects in their homeostatic lymphocyte profiles

To assess the extent of the role PSGL-1 plays in a steady state, we compared the baseline numbers of T cell subsets in WT and PSGL-1-deficient mice. CD4+ and CD8+ T cells of PSGL-1null mice were both significantly reduced in the peripheral blood by 44% and 45% respectively compared to WT mice (Figure 3.1A). However, analysis of CD4+ and CD8+ T cell numbers in lymph nodes and spleen showed that while there may be a trend for reduced numbers, this was not statistically significant (Figure 3.1B&C). B cell numbers were also found to be comparable in lymph nodes, spleen and peripheral blood in WT and PSGL-1null mice. Despite this trend, lymph nodes in PSGL-1null mice tended to be slightly bigger in size in age- and sex-matched WT mice.

The observed T cell lymphopenia in the peripheral blood prompted a more detailed analysis of the naïve and memory T cell subsets to determine whether all subsets were similarly affected. Unexpectedly, CD4+ and CD8+ naïve T cells, defined as CD44low L-selectinhigh, were reduced by 98% and 95% respectively in the peripheral blood (Figure 3.2A). In lymph nodes, naïve CD4+ and CD8+ T cells were reduced by 42% and 54% respectively whereas in the spleen there was a trend for a decrease in naïve T cell numbers, but it was not significant
(Figure 3.2B&C). In some experiments, central and effector memory T cells (CD44$^{\text{high}}$) showed a trend to be elevated in PSGL-1$^{\text{null}}$ mice but the numbers were inconsistent between data sets.

Figure 3.1: Lymphocyte numbers in lymph nodes and spleen are normal in PSGL-1$^{\text{null}}$ mice but are reduced in peripheral blood. Lymphocytes were isolated from A) peripheral blood, B) lymph nodes and C) spleens of 8 week old WT and PSGL-1$^{\text{null}}$ mice. Cells were stained with CD4, CD8 and B220 mAbs and measured by flow cytometry. Numbers are representative of absolute numbers in each tissue, as measured by counting beads. Data represents at least 3 separate experiments. N = 3. Error bars represent standard deviation. * p<0.05, **p<0.01 using a Student's T-test.
**Figure 3.2:** Naïve T cells are reduced in peripheral blood and lymph nodes, but are normal in spleen. Lymphocytes were isolated from A) spleens, B) lymph nodes and C) peripheral blood of 8 week old WT and PSGL-1-null mice. Cells were stained with CD4, CD8, CD44 and L-selectin mAbs and measured by flow cytometry. Numbers are representative of absolute numbers in each tissue, as measured by counting beads. Data represents at least 3 separate experiments. N = 3. Error bars represent standard deviation and * p<0.05 using a Student's T-test.
3.2.2 PSGL-1\(^{\text{null}}\) mice have decreased levels of effector cells expressing activation markers

There are a number of potential reasons why PSGL-1\(^{\text{null}}\) mice may have reduced numbers of L-selectin-positive naïve T cells. One possibility is that naïve T cells that do not express PSGL-1 are unstable and therefore spontaneously undergo apoptosis. However, the fact that lymph nodes and spleen still contain substantial numbers of naïve T cells suggests they are inherently viable, albeit only in certain compartments. Another possibility is that PSGL-1\(^{\text{null}}\) T cells become activated, shed L-selectin and differentiate into effector cells. This scenario seems unlikely though since we did not see a large increase in L-selectin\(^{\text{low}}\) effector cells (Figure 3.2).

While we define naïve T cells to be L-selectin\(^{\text{high}}\), it is possible that PSGL-1\(^{\text{null}}\) T cells are functionally naïve but lose L-selectin and therefore fall into the L-selectin\(^{\text{low}}\) category that we classify as effector T cells. To examine this possibility, we used CD25 and CD69, two early activation markers expressed on proliferating, activated cells, to determine if there were differences in the effector T cell subsets, defined as L-selectin\(^{\text{low}}\) CD44\(^{\text{low}}\), between WT and PSGL-1\(^{\text{null}}\) mice. As shown in figure 3.3, there was a reduced percentage of early activated T cells in PSGL-1\(^{\text{null}}\) mice compared to WT mice. These data show that within the L-selectin\(^{\text{low}}\) CD44\(^{\text{low}}\) T cell subset there was a greater proportion of PSGL-1\(^{\text{null}}\) T cells that do not show signs of activation. Therefore, it is possible that naïve T cells are shedding L-

![Figure 3.3](image-url)
selectin but are not becoming bona fide activated effector cells and yet appear as effector cells in our analyses based on the parameters we use to measure T cell subsets. However, there was no compensatory increase in L-selectin\textsubscript{low} effector T cell subset numbers in the peripheral blood of PSGL-1\textsuperscript{null} mice which would be expected if naïve T cells were shedding L-selectin and falling into the effector T cell gate. In fact, CD4+ effector T cell numbers in PSGL-1\textsuperscript{null} mice were even decreased compared to WT mice. Therefore, the loss of L-selectin\textsuperscript{high}CD44\textsuperscript{low} naïve T cell subsets in PSGL-1\textsuperscript{null} mice cannot be explained entirely by L-selectin shedding of naïve T cells.

3.2.3 T cell lymphopenia in PSGL-1\textsuperscript{null} mice is cell intrinsic

We then went on to ask whether the reduction of naïve T cells in PSGL-1\textsuperscript{null} mice is cell intrinsic or if it could be corrected in the presence of PSGL-1 expressed on other cells. This question was addressed by competitively reconstituting recipient mice with a 1:1 ratio of WT and PSGL-1\textsuperscript{null} bone marrow cells. IL-7R\textsuperscript{null} mice were used as recipients due to their inability to produce endogenous T cells and therefore are capable of T cell reconstitution after injection with bone marrow stem cells. The process of TCP homing into the thymus is partially dependent on the interaction of PSGL-1 on TCPs with P-selectin on thymic endothelium and therefore PSGL-1\textsuperscript{null} TCPs have a disadvantage at reconstituting the thymi of IL-7R\textsuperscript{null} mice compared to WT TCPs (Rossi et al., 2005). To eliminate this bias, we used IL-7R/P-selectin double knockout recipients in which both WT and PSGL-1\textsuperscript{null} TCPs can equally enter and reconstitute thymi. T cell subsets of reconstituted mice were analyzed in blood and lymph nodes 5 weeks after bone marrow injection. Percentages of L-selectin\textsuperscript{high}CD44\textsuperscript{low} naïve CD4+ and CD8+ T cells derived from WT and PSGL-1\textsuperscript{null} donor cells were comparable in the lymph nodes whereas in the blood, the numbers of PSGL-1\textsuperscript{null} L-selectin\textsuperscript{high}CD44\textsuperscript{low} naïve CD4+ and CD8+ T cells were considerably reduced compared to corresponding WT T cells (Figure 3.4). This suggests that the loss of naïve T cells in the blood of PSGL-1\textsuperscript{null} mice was due to a T cell intrinsic effect and not due to signals associated with general PSGL-1-deficiency in all hematopoietic cells.
Because CD4+ and CD8+ T cells are reduced in the peripheral blood of PSGL-1-null mice, we examined the half-lives of WT and PSGL-1-deficient T cells in the blood. To do this, we injected lymph node cells from PSGL-1-null and WT mice at a 1:1 ratio into WT recipients and tracked the ratios of cells remaining in blood over time. As seen in figure 3.5A, combined CD4+ and CD8+ PSGL-1-null T cells disappeared from the blood at a faster rate than WT T cells suggesting a shorter half-life of PSGL-1-null T cells in the peripheral blood. We repeated this experiment using single positive CD8+ thymocytes that are all naïve and do not have any memory T cells within this population, to eliminate the differences between naïve and memory T cell survival, as well as the possibility that selective pressures in the periphery may influence the inherent survival rates between WT and PSGL-1-null T cells. We used single positive CD8+ T cells from HY and HY/PSGL-1-null thymi, as these TCR transgenic mice have an increased number of these cells. Using CFSE labelling to track the cells in vivo, we again see PSGL-1-null CD44low L-selectinhigh naïve T cells are decreased in the peripheral blood of reconstituted mice. P-selectinnull IL-7Rnull mice reconstituted with equal parts WT and PSGL-1null bone marrow and analyzed 5 weeks later. Cells were stained with CD4, CD8, CD44, L-selectin, Thy1.1 and CD45.1 mAbs and measured by flow cytometry. Data represent at least three mice, and 4 separate experiments.
deficient CD8+ naïve thymocytes were lost at a faster rate than WT CD8+ thymocytes (Figure 3.5B). Here we note that PSGL-1null naïve T cells harvested from the thymus disappeared even quicker than PSGL-1null T cells harvested from lymph nodes.

3.2.5 PSGL-1 is required for efficient homing to secondary lymphoid organs

Because PSGL-1null T cells disappeared from the peripheral blood at a faster rate than WT T cells, we might expect that they would instead appear in SLOs at a higher rate. In contrast, a previous study looking at thymic progenitor migration in a parabiotic mouse model showed there were approximately 50% less PSGL-1null T cells in the lymph nodes compared to WT T cells whereas there were comparable numbers of each genotype in the spleen (Rossi et al., 2005). To elucidate how PSGL-1 may be playing a role in the movement of T cells from the blood to the SLOs, we used a short term competitive homing assay in which labelled lymphocytes from WT or PSGL-1null were injected i.v. at a 1:1 ratio into WT recipients and analyzed one hour later. Homing of PSGL-1null lymphocytes to lymph nodes was 49% less efficient than homing of WT lymphocytes whereas homing of PSGL-1null lymphocytes to Peyer's...
patches was reduced by 35% and homing to the spleen was reduced by only 10% (Figure 3.6A). In the peripheral blood, the numbers of WT and PSGL-1null lymphocytes were comparable one hour after cell injection, consistent with data in figure 3.5. Control experiments verified fluorescent labels did not affect homing properties of lymphocytes to lymph nodes (Data not

Figure 3.6: The effect of PSGL-1 on homing to SLOs is restricted to resting T lymphocytes and is dependent on expression levels. Competitive in vivo short-term homing of lymphocytes from (A) WT and PSGL-1null mouse donors in WT recipients; relative percentages of labelled lymphocytes in recipient peripheral blood (PB), lymph nodes (LN), Peyer's patches (PP) and spleen (Spl) were determined by flow cytometry after 1 h. Statistics are representative of difference between WT and PSGL-1null mice, (B) WT, PSGL-1heterozygote and PSGL-1null donors in WT recipients. Statistics are representative of difference between PSGL-1heterozygote and PSGL-1null T cells, (C) WT and PSGL-1null mouse donors in WT recipients, stained with CD4, CD8 and CD44 mAbs. Statistics are representative of difference between PB and LN of PSGL-1null T cells. * p < 0.05, ** p < 0.01 (Student's t-test). N= 3. Data are mean percent cells relative to WT and are representative of at least three independent experiments. Error bars represent standard deviation.
shown). Reduced homing was not due to decreased viability in the blood because cells were not lost in the blood after the short term one hour time point. To assess if PSGL-1 affects lymph node homing in a dose dependent manner, we measured WT, PSGL-1\textsuperscript{null} and PSGL-1\textsuperscript{hetero} lymph node cell homing. PSGL-1\textsuperscript{hetero} T cells express less PSGL-1 on the surface than WT T cells (data not shown.) PSGL-1\textsuperscript{hetero} lymphocytes consistently entered SLOs at a higher rate than PSGL-1\textsuperscript{null} lymphocytes, but less efficiently than WT lymphocytes (Figure 3.6B). We also looked at B cells and T cell subsets to determine whether all lymphocytes show a similar requirement for PSGL-1 for efficient lymph node homing. PSGL-1\textsuperscript{null} B cells showed no impairment in lymph node homing. CD4+ and CD8+ T cells showed a dependency on PSGL-1 for efficient T cell homing to lymph nodes, both in naïve and in memory subsets (Figure 3.6C).

Therefore, PSGL-1 appeared to be playing a role in efficient homing of CD4+ and CD8+ T cells to lymph nodes that is dose dependent.

### 3.2.6 T cell homing is not affected by CD43

PSGL-1 is a large, abundant glycoprotein found on the surface of most leukocytes. To determine whether loss of efficient homing is dependent on PSGL-1 specifically or whether it is the consequence of the loss of an abundant surface glycoprotein, we did competitive short-term homing assays with WT and CD43\textsuperscript{null} lymph node cells. CD43 is another large, abundant glycoprotein on T cells that carries extensive O-glycans and has an extended structure similar to PSGL-1 (Cyster et al., 1991). It has also been shown to be able to

**Figure 3.7: CD43 does not contribute to T cell homing.** Competitive in vivo short-term homing of lymphocytes from WT and CD43\textsuperscript{null} mouse donors; relative numbers of labelled lymphocytes in recipient peripheral blood (PB), lymph nodes (LN), Peyer’s patches (PP) and spleen (Spl) were determined by flow cytometry after 1 h. * p < 0.05 (Student’s t-test) N = 4 Data are mean percent cells relative to WT and are representative of at least three independent experiments. Error bars represent standard deviation.
bind to E-selectin in a Core 2 dependent manner (Matsumoto et al., 2005). Our homing assays show that loss of CD43 did not affect the ability of lymphocytes to enter SLOs (Figure 3.7). This would suggest that efficient short term T cell homing to SLOs was PSGL-1 specific and not generalized to a loss of abundant negatively charged cell surface glycoproteins.

3.2.7 PSGL-1 affects T cell homing in a selectin independent manner

The predominant function of PSGL-1 is as a selectin ligand (Ley and Kansas, 2004). P-selectin and E-selectin are traditionally found on the blood vessel endothelium of inflamed tissues (Janeway, 2005). However, these selectins have been shown to be present in HEVs under chronic inflammatory conditions (Andoh et al., 1996). We therefore speculated that PSGL-1 may be interacting with very low amounts of steady state E- and/or P-selectin expressed in the HEVs. To test this hypothesis, we performed competitive homing experiments with WT and PSGL-1 null lymphocytes in E-selectin null or P-selectin null recipients with the expectation that the PSGL-1-dependent SLO homing advantage of WT T cells would be lost if it was selectin dependent. We found that WT lymphocytes maintained their advantage over PSGL-1 null lymphocytes, suggesting that neither selectin was involved in the PSGL-1 mediated homing of T cells to SLOs (Figure 3.8A&B).

A further possibility whereby PSGL-1 may be acting as a selectin ligand in SLO homing is through a secondary tethering mechanism. L-selectin, found on leukocytes, is capable of enhancing T cell migration to areas of inflammation by binding PSGL-1 found on leukocytes already bound to the endothelium (Sperandio et al., 2003; Spertini et al., 1996). The PSGL-1 advantage in T cell homing may therefore be attributed to L-selectin. Since L-selectin is critical to lymphocyte homing and T cells cannot enter lymph nodes without it, it was not possible to use L-selectin null T cells as donor cells. However, C2GnT-1 is required for L-selectin (and P-selectin) ligand formation on PSGL-1. L-selectin binding to PSGL-1 can therefore be eliminated in the absence of C2GnT-1 while leaving L-selectin binding to PNAd intact (Ellies et al., 1998;
Martinez et al., 2005). We thus used C2GnT-1null and C2GnT-1null/PSGL-1null lymph node donor cells in our competitive homing studies. Our data shows that PSGL-1-deficient T cells maintained their disadvantage even when L-selectin (and P-selectin) binding to PSGL-1 is eliminated (Figure 3.8C).

Figure 3.8: PSGL-1-enhanced homing of T cells to SLOs in not dependent on selectin interaction. Competitive in vivo short-term homing of lymphocytes from WT and PSGL-1null mouse donors in (A) E-selectinnull recipients or (B) P-selectinnull recipients; relative numbers of labelled lymphocytes in recipient peripheral blood (PB), lymph nodes (LN), Peyer’s patches (PP) and spleen (Spl) were determined by flow cytometry after 1 h. Competitive in vivo short-term homing of lymphocytes from C2GnT-1null (C2null) and PSGL-1null/C2GnT-1null (PSGL-1null-C2null) in (C) WT recipients or (D) E-selectinnull recipients. Statistics are representative of difference between WT and PSGL-1null mice (A&B) or C2null and PSGL-1null.C2null mice (C&D). * p < 0.05, ** p < 0.01 (Student’s t-test). N= 4 (A,C&D); N=3 (B) Data are mean percent cells relative to WT and are representative of at least three independent experiments. Error bars represent standard deviation.
E-selectin has been shown to bind PSGL-1 even in the absence of C2GnT-1 (Goetz et al., 1997). Therefore, to eliminate the involvement of all three selectins in this particular competitive setup, we injected C2GnT-1null and C2GnT-1null/PSGL-1null lymphocytes into E-selectinnull recipients. The disadvantage of PSGL-1-deficient T cells remains, thereby illustrating that none of the selectins is involved in PSGL-1 enhanced homing (Figure 3.8D). These data show that PSGL-1 play a role in T cell homing to lymph nodes and reveal a novel function of this molecule that is independent of interactions with any of the selectins.

3.2.8 PSGL-1null T cells have lower chemotactic responses

T cell entry into SLOs is reliant on chemokine gradients to direct lymphocytes to HEVs. The secondary lymphoid chemokines CCL21 and CCL19 are constitutively presented on cell surfaces of the HEVs and are well known to be essential for T cell migration (Nakano and Gunn, 2001). In addition to their chemotactic role, upon binding with their receptor CCR7 on lymphocytes, they stimulate pathways involved in activation of integrins and other migration molecules (Shimonaka et al., 2003; Stein et al., 2000). A previous study showed that human PSGL-1 is capable of binding the chemokines CCL21, CCL28 and CCL27 and that binding to CCL27 occurs at the N terminus in a tyrosine sulfation-dependent manner (Hirata et al., 2004). Therefore, we speculated that PSGL-1 may be affecting T cell homing through an interaction with CCL21 and/or CCL19. To test this hypothesis, we set up a competitive transwell chemotaxis assay using fluorescently labelled WT and PSGL-1null lymphocytes. Our data show that PSGL-1null T cells had an approximate 30% decrease in migration compared to WT cells in response to CCL21 (Figure 3.9A). To exclude the possibility that this reduced chemotactic response was due to the loss of a large, abundant glycoprotein, we again tested CD43null lymphocytes and found that these cells were equally as capable of migrating toward CCL21 as WT lymphocytes (Figure 3.9B). In fact, CD43null lymphocytes seemed to migrate 5-8% more efficiently than WT lymphocytes. We also tested in vitro migration of lymphocytes in response to
CCL19. Our data show that PSGL-1\textsuperscript{null} lymphocytes demonstrated a similar decrease in migration in response to CCL19 as seen in response to CCL21 (Figure 3.9C).

CXCL12 is another important chemokine involved in lymphocyte homing in HEVs that binds to CXCR4 on the surface of lymphocytes. Therefore we used purified T cells to determine whether PSGL-1 may affect chemotactic responses to a non-CCR7 ligand, specifically CXCL12, to determine whether PSGL-1 is important for chemotaxis toward other SLO chemokines. Interestingly, data show the PSGL-1 effect was restricted to CCL21 and CCL19 as the response

![Graphs showing migration of T cells](image)

**Figure 3.9:** Lymphocytes expressing PSGL-1 migrate better against CCL21 and CCL19 but not CXCL12. Competitive in vitro transwell chemotaxis assays of lymphocytes from A) WT and PSGL-1\textsuperscript{null} and B) WT and CD43\textsuperscript{null} mouse donors against CCL21. Competitive in vitro transwell chemotaxis assays of T cells (>95%) from WT and (C) PSGL-1\textsuperscript{null} mice or lymphocytes from (D) L-selectin\textsuperscript{null} mice against 200nM CCL21, CCL19 and CXCL12. Statistics are representative of difference between WT and knockout cells. * p < 0.05 (Student's t-test). All experiments were done in triplicate and are representative of multiple experiments. Error bars represent standard deviation.
to both these chemokines was reduced approximately 30% for PSGL-1null T cells compared to WT T cells whereas both WT and PSGL-1null T cells migrated similarly in response to CXCL12 (Figure 3.9C).

A significantly large proportion of T cells in the lymph nodes of PSGL-1null mice have reduced levels of L-selectin (Figure 3.2B). Therefore we speculated that the decrease in L-selectin may influence the in vitro chemotactic response of PSGL-1null T cells. To examine this possibility, we compared the chemotactic responses of L-selectinnull and WT lymphocytes to CCL21. Our data show that there was no difference in migration between WT and L-selectinnull lymphocytes confirming that it is the loss of PSGL-1 and not the loss of L-selectin on PSGL-1null T cells that affected T cell migration (Figure 3.9D).

Our collaborator, Steven Rosen (UCSF), showed that the PSGL-1 4RA10 antibody blocks T cell homing to SLOs (Veerman et al., 2007). Similar to our data using PSGL-1null T cells, antibody inhibition of SLO entry was strongest in peripheral and mesenteric lymph nodes, followed by Peyer's Patches. There was a trend for decreased homing to spleen, but it was not statistically significant. These antibody inhibition data in conjunction with our chemotaxis assays suggest that the PSGL-1-dependent chemotaxis effect could be abrogated using PSGL-1 binding antibodies. To verify this hypothesis, we treated WT and PSGL-1null T cells with PSGL-1 mAb 4RA10 or control CD43 mAb S11 and carried out competitive chemotaxis assays with CCL21 and CCL19. As expected, mAb S11 did not have an effect on T cell migration toward either chemokine whereas PSGL-1 mAb 4RA10 reduced WT T cell migration to similar levels as was seen for migration of PSGL-1null T cells (Figure 3.10A&B). Chemotaxis of antibody treated WT cells was reduced to levels seen in PSGL-1null T cells demonstrating that mAb blockade of PSGL-1 was equally as effective at eliminating the PSGL-1-dependent homing effect as no PSGL-1 surface expression. In conclusion, we determined that binding of CCL21 and/or CCL19 to PSGL-1 on T cells was required for efficient homing to SLOs.
P-selectin binding to PSGL-1 has been shown to mediate integrin activation and slow rolling through downstream signalling (Wang et al., 2007). Therefore, we considered the possibility that PSGL-1 signalling may contribute to increased T cell migration in response to CCL21 and CCL19. PSGL-1 has been linked to various signalling pathways, many of which overlap with CCR7 signalling pathways. We targeted Syk as it is a downstream target of PSGL-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Figure 3.10: Lymphocyte migration is blocked by anti-PSGL-1 antibody but not by piceatannol.** Competitive *in vitro* transwell chemotaxis assay of the migration of WT T cells and GFP-labeled PSGL-1 null T cells toward (A & C) 200 nM CCL21 or (B) 200 nM CCL19 after incubation of cells with mAb 4RA10 (to PSGL-1) or mAb S11 (to CD43). (C) Indicated amounts of piceatannol (PIC) were added to the cells before transfer into the top of transwell. Statistics are representative of difference between WT and knockout cells. * p < 0.05 (Student's t-test). All experiments were done in triplicate and are representative of multiple experiments. Error bars represent standard deviation.
1 as there are no studies directly linking Syk to CCR7 signalling. We added piceatannol, a Syk-selective tyrosine kinase inhibitor, to migrating T cells to see if it could abrogate the difference between WT and PSGL-1 null T cell migration toward CCL21. Piceatannol has also been shown to inhibit ZAP-70, a Syk homologue found in T cells (Soede et al., 1998). Our data show that piceatannol neither diminished the level of overall T cell migration toward CCL21, nor did it eliminate the advantage WT T cells had over PSGL-1 null T cells in chemokine mediated migration (Figure 3.10C). This would suggest that neither Syk nor ZAP-70 play a role in PSGL-1-dependent T cell migration in response to CCL21.

3.2.9 CCL21 binds T cells in a PSGL-1-dependent manner

CCL21 and CCL19 have been shown to bind CCR7 on T cells as well as cell surface–expressed GAGs, including heparan sulfate and chondroitin sulphate found on endothelial cells (Cyster, 2000; Patel et al., 2001). More recently, recombinant human PSGL-1 has been shown to bind CCL21 (Hirata et al., 2004). To determine whether primary murine T cells were capable of binding CCL21 in a PSGL-1-dependent manner, we synthesized murine CCL21 biotinylated at the N terminus and incubated the modified chemokine with lymph node cells at 37°C for 2 hours. WT CD4+ and CD8+ T cells bound higher levels of CCL21 than PSGL-1 null CD4+ and CD8+ T cells suggesting that PSGL-1 accounts for some cell surface binding of CCL21, in addition to CCR7 (Figure 3.11A). Residual binding of CCL21 is most likely to CCR7 on the surface of cells. In contrast to T cells, PSGL-1 status did not affect CCL21 binding to B cells as expected since B cells express very low levels of PSGL-1 on their surface.

When WT T cells were treated with PSGL-1 antibody 4RA10, the levels of CCL21 binding were reduced on both CD4+ and CD8+ T cells to similar levels seen on PSGL-1 null T cells whereas CCL21 binding on WT B cells or to PSGL-1 null T or B cells was not affected by 4RA10 antibody (Figure 3.11B&C). Antibody treatment with control CD43 antibody also showed no effect. Collectively, our data show that 4RA10 inhibited CCL21 binding to PSGL-1.
Figure 3.11: PSGL-1 binds CCL21. A) Flow cytometry of WT and PSGL-1<sup>null</sup> lymphocytes incubated for 2 h with a mixture of biotin-CCL21 and streptavidin-FITC, then co-stained with anti-CD4, anti-CD8 or anti-B220. Flow cytometry of (B) WT lymphocytes and (C) PSGL-1<sup>null</sup> lymphocytes incubated for 30 min with mAb 4RA10 (to PSGL-1) or mAb S11 (to CD43) before 2 h of incubation with CCL21-FITC followed by co-staining with CD4, CD8 or B220 mAbs. (D) Flow cytometry of WT and PSGL-1<sup>null</sup> lymphocytes incubated for 2 h with a mixture of biotin-CCL21 and streptavidin-FITC in the presence of 0.017% sodium azide, then co-stained with anti-CD4, anti-CD8 or anti-B220. Data are representative of four independent experiments.
Furthermore, they provided an indication of the location of CCL21 binding to PSGL-1. 4RA10 has been mapped to the N terminus of PSGL-1 and has been shown to block P-selectin binding (Thatte et al., 2002). Therefore we would expect that CCL21 binds PSGL-1 at the N-terminus in a similar location where selectin binding occurs.

CCL21 binding assays were done under similar conditions as chemotaxis assays which caused T cells to internalize chemokine receptors. This resulted in a biphasic pattern of chemokine binding in which a large number of cells were CCL21\textsuperscript{dim}. To prevent chemokine internalization, we performed CCL21 binding assays in the presence of sodium azide resulting in monophasic levels of bound CCL21 (Figure 3.11D). Consistently, WT T cells still bind CCL21 at a higher frequency than PSGL-1\textsuperscript{null} T cells.

### 3.2.10 PSGL-1-enhanced chemotaxis is lost on activated T cells

Activated T cells respond to different chemokines than resting T cells such as CXCL9, CXCL10 and CXCL11. To determine whether PSGL-1 also has an effect on activated T cell migration in response to inflammatory chemokines, we activated both WT and PSGL-1\textsuperscript{null} T cells with the mitogen concanavalin A and monitored their migration to CXCL9 and CXCL11 in addition to CCL21. Activated T cells still migrated efficiently in response to CCL21 while WT T cells no longer had an advantage over PSGL-1\textsuperscript{null} T cells (Figure 3.12A). There were also no differences in migration to CXCL9 and CXCL11, suggesting that PSGL-1 only plays a role in migration of resting T cells in response to CCL21 and CCL19.

Upon activation, T cells upregulate glycosyltransferases that mediate carbohydrate modifications on PSGL-1 required for selectin binding. We used C2GnT-1 knockout mice that cannot form sLe\textsuperscript{x} on core 2 O-glycans to determine if this modification blocks the binding site for CCL21 and is responsible for eliminating the role of PSGL-1 in migration of activated T cells (Ley and Kansas, 2004). Under resting conditions, C2GnT-1\textsuperscript{null} T cells were not impaired in T
cell migration in response to CCL21 (Figure 3.12B). In fact, there was a general increase of overall chemotaxis compared to both WT and PSGL-1\textsuperscript{-null} T cells that do express C2GnT-1.

However, ConA activated PSGL-1\textsuperscript{-null} T cells migrated at a comparable rate to WT T cells. If activated T cells were incapable of expressing branched core 2 O-glycans, PSGL-1-deficient T cells were again at a disadvantage. Interestingly, activated C2GnT-1\textsuperscript{-null} T cells migrated twofold better than activated T cells from either WT or PSGL-1\textsuperscript{-null} donors. These data would suggest that CCL21 bound to PSGL-1 at the N-terminus and that this binding was abrogated upon activation of T cells due to core 2 O-glycan branching. This allows for an effective mechanism to prevent activated T cells from responding to homeostatic chemokines and re-entering SLOs.

![Figure 3.12: Activated T cells do not have a PSGL-1-dependent chemotactic advantage.](image)

**Figure 3.12: Activated T cells do not have a PSGL-1-dependent chemotactic advantage.** Competitive in vitro transwell chemotaxis assay of (A) the migration of WT T cells and PSGL-1\textsuperscript{-null} T cells toward 200 nM CCL21, CXCL11 or CXCL9 4 days after activation with ConA or (B) the migration of WT and PSGL-1\textsuperscript{-null} or C2null and C2null/PSGL-1\textsuperscript{-null} either naïve or after activation with ConA. Statistics are representative of difference between PSGL-1 expressing and PSGL-1 non-expressing cells. *p < 0.05 (Student's t-test). Migration was done in triplicate and data is representative of three independent experiments and error bars represent standard deviation.
3.2.11 PSGL-1<sup>null</sup> T cells are retained in lymph nodes

PSGL-1<sup>null</sup> T cells had a decreased rate of SLO entry yet the number of T cells in lymph nodes was not significantly reduced in PSGL-1<sup>null</sup> mice. Therefore, we speculated that T cells may also have a decreased rate of lymph node egress. To test this hypothesis, we preloaded lymph nodes with a mixture of HY and HY/PSGL-1<sup>null</sup> thymocytes and then blocked further lymph node entry using anti-integrin antibodies. Lymph node cells of one set of mice were analyzed after preloading to determine initial ratios at time point 0 hr. After 24 and 48 hours, lymph node cells from integrin mAb treated mice were analyzed to determine differences in ratios between WT and PSGL-1<sup>null</sup> donor T cells after egress over time. The ratio of PSGL-1<sup>null</sup> CD8<sup>+</sup> T cells to WT CD8<sup>+</sup> T cells increased over time in integrin mAb treated mice indicating PSGL-1<sup>null</sup> T cells were retained longer in lymph nodes than WT T cells (Figure 3.13).

![Figure 3.13: PSGL-1 is needed for efficient T cell egress from lymph nodes. CD8<sup>+</sup> T HY and HY/PSGL-1<sup>null</sup> thymocytes were injected into WT recipients and lymph node entry blocked with anti-integrin antibodies. Mice were sacrificed at various time points and the ratio of PSGL-1<sup>null</sup>:WT T cells were measured by flow cytometry. Statistics are representative of difference between the initial time point and the time points that follow. * p < 0.05 (Student's t-test). N=4. Data are ratio of PSGL-1<sup>null</sup>:WT and are representative of at least three independent experiments. Error bars represent standard deviation.]

3.2.12 PSGL-1<sup>null</sup> CD8<sup>+</sup> T cells spontaneously proliferate <i>in vivo</i> after 5 weeks

Another compensatory mechanism that may explain why numbers of T cells in SLOs are within a normal range in PSGL-1<sup>null</sup> mice is homeostatic proliferation, a process that occurs when T cell numbers diminish in the periphery and the body compensates by stimulating T cells to divide and expand to fill the depleted T cell compartment. We used two common homeostatic
proliferation models, IL-7R<sup>null</sup> mice and sub-lethally irradiated WT mice to compare WT and PSGL-1<sup>null</sup> T cell repopulation in vivo. We hypothesized that lymphopenia in PSGL-1<sup>null</sup> mice may also stimulate T cells to expand and therefore used non-irradiated PSGL-1<sup>null</sup> mice and non-irradiated WT mice controls as a third homeostatic proliferation model. Lymph node cells from WT and PSGL-1<sup>null</sup> mice were stained with CFSE and injected i.v. into IL-7R<sup>null</sup> mice and CFSE dilution was measured in T cells recovered from spleen 4 days later. Spleens were analyzed, as opposed to lymph nodes in all other studies, because lymph nodes in IL-7R<sup>null</sup> mice are severely reduced in size and difficult to acquire. As seen in figure 3.14A, T cells from both WT and PSGL-1<sup>null</sup> divided at the same rate after this relatively short time span. Another widely used assay to measure homeostatic proliferation is based on sublethally irradiated mice. Here, we irradiated WT mice with 350 rad and 24 hours later they were injected with CFSE labelled lymph node cells from both WT and PSGL-1<sup>null</sup> donors. After 7 days, T cells from lymph nodes were analyzed (Figure 3.14B). CFSE dilution profiles show that both WT and PSGL-1<sup>null</sup> T cells proliferated equally well in irradiated hosts. In CD8+ T cells, there was a trend for PSGL-1<sup>null</sup> T cells to proliferate somewhat faster, but dilution rates were still comparable to WT T cells.

Both these models show that WT and PSGL-1<sup>null</sup> T cells have a similar capacity to proliferate and repopulate the depleted T cell compartments in lymphopenic mice. We thus speculated that the T cell lymphopenia observed in PSGL-1<sup>null</sup> mice may lead to homeostatic expansion of T cells and thereby accounting for the normal levels of total T cell numbers. To test this, we co-injected both WT and PSGL-1<sup>null</sup> lymph node cells at a 1:1 ratio into either non-irradiated WT or PSGL-1<sup>null</sup> recipients hypothesizing that after 5 weeks, T cells injected into PSGL-1<sup>null</sup> recipients would undergo cell division whereas T cells injected into WT recipients would not. The results were surprising in that PSGL-1<sup>null</sup> donor cells showed a clear increase in proliferation after 5 weeks compared to WT donor cells, irrespective of whether cells were transferred into WT or PSGL-1<sup>null</sup> recipients (Figure 3.14C). To look at proliferation over time, we
did a time course to determine at what time point CD8+ T cell proliferation can be seen. Within 2 weeks, division could be measured and levels increased gradually up to 5 weeks (Figure 3.15).

Lymphopenia-induced proliferation (LIP) occurs when lymphocyte numbers are reduced below a particular threshold (Osborne et al., 2011). Our data suggest that the PSGL-1<sup>null</sup> mouse is not sufficiently lymphopenic to induce LIP in T cells but rather that PSGL-1<sup>null</sup> T cells are undergoing spontaneous proliferation independently of their environment.
Figure 3.14: PSGL-1null CD8+ T cells spontaneously proliferate in vivo after 5 weeks. Lymph node cells from WT and PSGL-1null mice were labelled with CFSE and injected into indicated recipient mice. (A) Spleens of IL7Rnull mice were analyzed for CFSE dilution after 4 days. (B) Lymph nodes were analyzed after 7 days and (C&D) after 35 days. Donor cells were identified using Thy1 and CD45 congenic markers. Data shown are representative of at least three mice per experiment, and are representative of at least three independent experiments.
Figure 3.15: Spontaneous proliferation of PSGL-1<sub>null</sub> T cells occurs slowly. Lymph node cells from WT and PSGL-1<sub>null</sub> mice and labelled with CFSE before injected intravenously into WT mice. Lymph nodes were analyzed after indicated time points for CFSE expansion using CD4 and CD8 mAbs. Donor cells were identified using Thy1 and CD45 congenic markers. Data shown are representative of at least three mice per experiment, and are representative of at least two independent experiments.
3.2.13 WT T cells survive better than PSGL-1null T cells after adoptive transfer

In 5 week homeostatic expansion assays, we also measured the ratio of WT and PSGL-1null T cells recovered from lymph nodes. While cells were injected at a 50:50 ratio, over time the cell ratios skewed to approximately 60:35 WT to PSGL-1null CD4+ T cells and 70:25 WT to PSGL-1null CD8+ T cells respectively (Figure 3.16). Seeing as CD8+ PSGL-1null T cells undergo homeostatic proliferation, we would therefore expect their numbers to increase and the ratios to skew in favour of PSGL-1null T cells. Nevertheless, in light of the inability of PSGL-1null T cells to enter lymph nodes efficiently as well as a decreased half-life in peripheral blood, it appears that the proliferation of PSGL-1null T cells was not sufficient to compensate for the general loss of PSGL-1null T cells.

Figure 3.16: PSGL-1null T cells disappear more rapidly after adoptive transfer in comparison to WT T cells. Lymph node cells from WT and PSGL-1null mice were labelled with CFSE and injected into WT mice. Lymph nodes were analyzed for CFSE expansion after 5 weeks using CD4 and CD8 mAbs. Donor cells were identified using Thy1 and CD45 congenic markers. Statistics are representative of the difference between WT and PSGL-1null T cells. *** p < 0.001 (Student's t-test). N=3. Data are mean percent cells relative to WT and are representative of at least three independent experiments.
3.2.14 PSGL-1<sup>null</sup> T cell proliferation is cell intrinsic as well as partially dependent on lymph node resident time

We have previously shown that PSGL-1<sup>null</sup> T cells have an increase in residence time in the lymph nodes (Figure 3.13). Homeostatic cytokines such as IL-7, IL-15 and IL-2 are found in lymph nodes where homeostatic proliferation is known to occur (Surh and Sprent, 2008). To determine whether the increase in PSGL-1<sup>null</sup> T cell proliferation is due to increased lymph node residence time, we used 4'-deoxypyridoxine (DOP), a vitamin-B<sub>6</sub> antagonist that blocks T cell egress from lymph nodes (Schwab et al., 2005). We injected WT and PSGL-1<sup>null</sup> lymph node cells into mice treated with DOP, causing both WT and PSGL-1<sup>null</sup> T cells to become trapped in lymph nodes and hence reside in the lymph nodes for equal lengths of time. After 5 weeks of treatment, CFSE dilution analysis showed that PSGL-1<sup>null</sup> CD8<sup>+</sup> T cells continued to have a clear proliferative advantage over WT CD8<sup>+</sup> T cells even when spending equivalent lengths of time in the same lymph node microenvironment (Figure 3.17A). When comparing the percentage of dividing WT and PSGL-1<sup>null</sup> T cells, PSGL-1<sup>null</sup> T cells consistently proliferated more than WT donor CD8<sup>+</sup> T cells (Figure 3.17B). As expected, both WT and PSGL-1<sup>null</sup> donor T cells expanded somewhat more in DOP-treated recipient mice however this difference was not statistically significant. Nevertheless, our data suggest that lymph node residence time may be a further factor contributing to increased expansion of PSGL-1<sup>null</sup> CD8<sup>+</sup> T cells. Thus, the increased proliferation of PSGL-1<sup>null</sup> CD8<sup>+</sup> T cells seen <i>in vivo</i> can be attributed both to an increase in lymph node residence time as well as a cell intrinsic proliferative advantage of PSGL-1<sup>null</sup> CD8<sup>+</sup> T cells.
Figure 3.17: PSGL-1\textsuperscript{null} T cells divide more rapidly than WT T cells, even when treated with DOP to prolong their residence time in the lymph nodes. Lymph node cells from WT and PSGL-1\textsuperscript{null} mice and labelled with 2 \textmu M CFSE before injected intravenously into WT mice. Recipient mice were given continuous DOP treated drinking water. Lymph nodes were analyzed after 5 weeks for CFSE expansion using CD4 and CD8 mAbs. Donor cells were identified using Thy1 and CD45 congenic markers. A) Histograms comparing CD4\textsuperscript{+} and CD8\textsuperscript{+} WT and PSGL-1\textsuperscript{null} T cells in treated or untreated mice. B) Dot plots representing a comparison of the percentage of PSGL-1\textsuperscript{null} CD8\textsuperscript{+} T cells divided against percentage of WT CD8\textsuperscript{+} T cells divided within a single recipient. Data shown are pooled from three independent experiments.
3.2.15 PSGL-1null T cells show increased proliferation rates to homeostatic cytokines in vitro

Homeostatic cytokines play an important role in homeostatic proliferation. IL-7, IL-15 and IL-2 have all been implicated to promote T cell survival when found in low concentrations and promote T cell proliferation when found in high concentrations (Sprent and Surh, 2003). To determine whether the spontaneous in vivo PSGL-1null T cell proliferation we have observed could be linked to a difference in response to these homeostatic cytokines, we examined the proliferative response of T cells exposed to various cytokines in vitro. CFSE labelled lymph node cells from WT and PSGL-1null mice were placed at a 1:1 ratio in culture with various cytokines. After 4 days in culture, CD4+ T cells did not show any CFSE dilution to any stimulus (data not shown). CD8+ T cells did not proliferate in response to IL-7 after 4 days (data not shown) but they did proliferate in response to IL-15, IL-2 and IL-4 starting at day 3 and showed substantial amounts of proliferation by day 4 in a dose dependent manner (Figure 3.18A,B&C). IL-15 stimulated T cells most efficiently as there were approximately 4 fold more total T cells recovered when cultured with IL-15 than when cultured with IL-2 or IL-4. All three cytokines belong to the γc receptor family. To verify whether increased PSGL-1 dependent proliferation was restricted to this family of receptors we also looked at response to IL-6 and IL-12 but the resting cells did not proliferate (data not shown). We also tested proliferation of T cells in response to CCL21 or CCL19 as these chemokines have been linked to PSGL-1 in T cell homing, however they did not stimulate cells to proliferate (data not shown).
A)

Medium alone  1 ng/mL  10 ng/mL  50 ng/mL

Day 2

Medium alone  1 ng/mL  10 ng/mL  50 ng/mL

Day 3

Medium alone  1 ng/mL  10 ng/mL  50 ng/mL

Day 4

CFSE

WT
PSGL-1null
Unstained

IL-15

WT
PSGL-1null

Unstained

# cells x 10^3

0 200 400 600 800 1000 1200

Medium Alone  1 ng/mL  10 ng/mL  50 ng/mL

WT
PSGL-1null

Unstained
B

Medium alone  1 ng/mL  10 ng/mL  50 ng/mL

Day 2

Medium alone  1 ng/mL  10 ng/mL  50 ng/mL

Day 3

Medium alone  1 ng/mL  10 ng/mL  50 ng/mL

Day 4

CFSE

WT
PSGL-1/null
Unstained

IL-2
C Medium alone 1 ng/mL 10 ng/mL 50 ng/mL

Day 2

Medium alone 1 ng/mL 10 ng/mL 50 ng/mL

Day 3

Medium alone 1 ng/mL 10 ng/mL 50 ng/mL

Day 4

CFSE

WT
PSGL-1\textsuperscript{null}
Unstained

IL-4
CD4+ memory T cells have moderate expression levels of IL-15Rα and CD4+ naïve T cells do not express IL-15Rα at all whereas CD8+ memory T cells have been shown to express high levels of IL-15Rα on their surface compared to CD8+ naïve T cells (Zhang et al., 1998). Due to the increased surface expression levels of IL-15Rα, CD8+ memory T cells have been shown to respond quicker to IL-15 than CD8+ naïve T cells (Cho et al., 2007). To eliminate the possibility that differences in memory and naïve T cell ratios in WT and PSGL-1null lymph node preparations skew our data, we purified naïve and memory CD8+ T cells based on CD44 expression. Our data show that memory T cells proliferate quicker than naïve T cells, as expected, but also that both memory and naïve PSGL-1null CD8+ T cells proliferated more rapidly in the presence of IL-15 than corresponding WT CD8+ T cells (Figure 3.19).

Sorted T cells with no cytokine stimulus did not survive at all (data not shown).
Whole lymph node preparations were used in our initial in vitro proliferation experiments. In these cultures, dendritic cells and other APCs were also present with T cells. It is possible that these APCs contribute to in vitro proliferation through MHC engagement. To rule out such a possibility in the experiment described above, we purified T cells expressing CD8β to exclude dendritic cells, of which some subsets are known to express CD8α. It is clear that IL-15 was capable of inducing proliferation in the absence of APCs (Figure 3.19). Nevertheless, we cannot exclude the possibility that the TCR on CD8+ T cells were engaging with MHC-Class I on neighbouring CD8+ T cells and stimulating each other.

These data collectively show that PSGL-1null CD8+ T cells were hyper-proliferative in response to in vitro stimulation with IL-15, IL-4, IL-2 but not IL-7, IL-6, IL-12, CCL21 or CCL19. This proliferative advantage occurred in both naïve and memory T cells and was not dependent on interactions with APCs, suggesting it is cell intrinsic.

3.2.16 Proliferating T cells upregulate memory and activation markers

T cells stimulated with IL-15 and IL-2 have been shown to become memory phenotype cells, a subset of T cells that display memory markers, particularly CD44, but have not undergone activation or stimulation with their cognate antigen (Sprent et al., 2008). We used various cell markers to characterize CD8+ T cells after stimulation in vitro with IL-15. Figure 3.20A shows increased expression levels of CD44 on CD8+ T cells stimulated with IL-15 compared to the no cytokine control in both WT and PSGL-1null T cells. The differences seen between WT and PSGL-1null T cells were due to the difference in proliferation rates. When gated on dividing cells alone, the levels of CD44 expression were equal. To confirm the memory phenotype, we also looked at another memory marker on CD8+ T cells, Ly6c. After three days, IL-15 also increased Ly6c expression (Figure 3.20B). CD69, an early activation marker, also showed an increase in expression on proliferating cells (Figure 3.20C). This was surprising as we did not expect that T cells could be activated with IL-15 alone, even in the presence of
APCs. However, other studies have shown that homeostatic proliferation can induce a variety of phenotypes, including an activated phenotype with increased CD69 expression (Jameson, 2002).
Figure 3.20: CD8+ T cells upregulate CD44, Ly6c and CD69 after stimulation with IL-15. WT and PSGL-1\textsuperscript{null} lymph node cells were mixed, labelled with CFSE and placed in culture with medium alone, 10 ng/mL or 50 ng/mL IL-15 for 3 days, then stained with (A) CD44, (B) Ly6c and (C) CD69 mAbs. Data is shown as total viable CD8+ T cells based on PI staining, except where “divided cells alone” are labelled, having been gated on CFSE diminished cells. In WT and PSGL-1\textsuperscript{null} figures, data represents the shift between cytokine concentrations. All experiments were done in triplicate and are representative of multiple experiments.
3.2.17 There are no differences in cytokine receptor expression on WT and PSGL-1null T cells

IL-15 binds to the IL-15R heterotrimer complex consisting of IL-15Rα, IL-2Rβ and the γc. IL-2 binds to IL-2Rα of the IL-2R heterotrimer complex that shares IL-2Rβ and γc with the IL-15R. IL-4R consists of a heterodimer of IL-4α and the γc while IL-7R consists of a heterodimer of IL-7Rα and the γc. Both IL-15 and IL-2 have been known to signal through IL-2Rβ and γc in absence of their alpha chains when high enough concentrations of cytokines are present.

To determine whether increased T cell proliferation in PSGL-1null T cells is caused by increased expression of cytokine receptors, we measured the cell surface expression of IL-15Rα, IL-2Rα, IL-2Rβ and γc on resting T cells in the lymph nodes of WT and PSGL-1null mice. There were no differences in the expression of these receptor subsets on CD8+ T cells between WT and PSGL-1null T cells (Figure 3.21A). When T cells were stimulated in vitro with IL-15, the level of cell surface expression of all receptors increased compared to unstimulated controls. It appeared that PSGL-1null T cells expressed higher levels of cytokine receptors at certain concentrations of cytokine exposure, however these differences were caused by a difference in proliferation rate and when gated on dividing cells alone, the difference between the two cell types disappeared suggesting there were no statistically significant differences in expression of γc, IL-15Rα and IL-2RA between WT and PSGL-1null T cells (Figure 3.21B,D,E). There was a small increase in the expression levels of IL-2Rβ on PSGL-1null T cells compared to WT T cells (Figure 3.21C). The difference was significant when measured by mean fluorescence intensity (p=0.02) on stimulated cells but not on unstimulated cells (p=0.08). Nevertheless, the difference was small compared to the shift between T cells that have been stimulated with IL-15 compared to those that have not. Therefore, it is difficult to speculate whether this shift is of any relevance to the phenomenon we see, particularly since we see no difference on resting T cells from lymph nodes.
To conclude, PSGL-1\textsuperscript{null} T cells in lymph nodes did not express increased levels of cell surface receptors needed for IL-15 and IL-2 signalling. They also did not show increased levels of cell surface receptors after IL-15 stimulation, with the possible exception of IL-2Rβ.
3.2.18 CD8+ T cells and not NK, NK T cells or B cells proliferate in response to IL-15 in \textit{in vitro} proliferation assays

IL-2 and IL-15 has been shown to stimulate proliferation of NK cells \textit{in vitro} from spleen preparations (Carson et al., 1994; Henney et al., 1981). While CD8α has been shown to not be expressed on murine NK cells, it is present on certain subsets of NK T cells. Therefore, we used the NK cell marker NK1.1 in addition to CD8α to separate out various T cell and NK cell subsets. After 3 days, there were no viable NK cells (NK1.1+ CD8-) or NK T cells (NK1.1+ CD8+) in any of the cultures but there was a large percentage of CD8+NK1.1- T cells (Figure 3.21).

**Figure 3.21:** WT and PSGL-1null mice have equal levels of γc, IL-15Rα, IL-2Rβ, and IL-2Rα on CD8+ T cells. (A) Lymph nodes were extracted from WT and PSGL-1null mice and analyzed with indicated antibodies by flow cytometry. (B-E) WT and PSGL-1null lymph node cells were mixed, labelled with CFSE and placed in culture with medium alone, 10 ng/mL or 50 ng/mL IL-15 for 3 days, then stained with (B) γc, (C) IL-2Rβ, (D) IL-15Rα and (E) IL-2Rα. Donor cells were identified using Thy1 congenic markers. Data is shown as total CD8+ viable T cells based on PI staining, except where “divided cells alone” are labelled, having been gated on CFSE diminished cells. In WT and PSGL-1null figures, data represents the shift between cytokine concentrations. All experiments were done in triplicate and are representative of multiple experiments.
3.22A). To note, there were NK1.1+ cells present in culture but they were all PI positive and therefore no longer viable. We measured proliferation of B cells but they did not divide in response to IL-15 (Figure 3.22B). Therefore, CD8+ T cells were the primary lymph node cell subset that proliferated in vitro in response to IL-15 stimulation and made up the majority of the cells remaining after 3 days.

Figure 3.22: IL-15 stimulation does not produce large amounts of NK or NK T cells, nor causes B cells to proliferate. WT and PSGL-1null lymph node cells were mixed, labelled with CFSE and placed in culture with medium alone, 10 ng/mL or 50 ng/mL IL-15 for 3 days, then stained with and gated by (A) CD8α and NK1.1 or (B) B220. Donor cells were identified using Thy1 congenic markers. Data is shown as total viable cells based on PI staining. All experiments were done in triplicate and are representative of multiple experiments. Error bars represent standard deviation.
3.2.19 L-selectin is shed from proliferating cells in response to IL-15 stimulation

L-selectin is known to be shed from proliferating cells in response to activation (Kishimoto et al., 1989). To determine if L-selectin is also shed in response to IL-15 stimulated proliferation, we measured L-selectin levels on CD8+ T cells. Data shows that dividing cells in response to IL-15 also had reduced levels of L-selectin on their surface compared to unstimulated controls (Figure 3.23). This offers a possible explanation for the reduced levels of L-selectin we observed on T cells in peripheral blood and lymph nodes of PSGL-1null mice.

3.2.20 Syk does not play a role in PSGL-1 enhanced proliferation to IL-15

PSGL-1 is known to signal via the molecule Syk (Urzainqui et al., 2002). Syk has also been linked to IL-15R signalling in neutrophils (Ratthe and Girard, 2004). To determine if PSGL-1 signalling through Syk may influence proliferation in CD8+ T cells, we used the Syk inhibitor piceatannol. Since PSGL-1null T cells have increased proliferation in response to IL-15, we would speculate that phosphorylation of Syk would have a negative regulatory effect on proliferation and that addition of piceatannol would cause WT T cells to proliferate at the same rate as PSGL-1null T cells. As seen in figure 3.24, addition of piceatannol inhibited proliferation of both WT and PSGL-1null T cells, suggesting that it does not play a role in PSGL-1-enhanced proliferation, but rather, inhibits Syk dependent IL-15R signalling. Since piceatannol also inhibits ZAP-70 signalling, we cannot exclude the possibility that it is ZAP-70 that plays a role in IL-15.
signalling. Differences between WT and PSGL-1\textsuperscript{null} T cell proliferation, however, remained when proliferation was only partially blocked at 10 ng/mL of piceatannol treatment, suggesting that PSGL-1-dependent proliferation was independent of Syk and/or ZAP-70 phosphorylation.

![Figure 3.24: Inhibition of Syk reduces proliferation of CD8+ T cells, in WT and PSGL-1\textsuperscript{null} T cells. WT and PSGL-1\textsuperscript{null} lymph node cells were mixed, labelled with CFSE and placed in culture with IL-15 for 3 days with or without piceatannol added daily. T cells were analyzed by flow cytometry and donor T cells distinguished using Thy1 congenic markers. Data is shown as CD8+ viable cells based on PI staining. All experiments were done in triplicate and are representative of three independent experiments.](image)

3.2.21 PSGL-1\textsuperscript{null} T cells show a small increase in proliferation compared to WT T cells in response to ConA

To determine whether enhanced proliferation of PSGL-1\textsuperscript{null} CD8+ T cells was unique to stimulation of resting T cells by homeostatic cytokines, we examined the proliferation response of ConA activated T cells. Both CD8+ WT and PSGL-1\textsuperscript{null} T cells showed a rigorous proliferative response to mitogen activation. After two days, there appeared to be a greater percentage of PSGL-1\textsuperscript{null} T cells that had undergone four cell divisions compared to WT T cells (Figure 3.25). However, the ratio of WT to PSGL-1\textsuperscript{null} T cells remained similar to that of the non-stimulated control suggesting that PSGL-1\textsuperscript{null} T cells did not proliferate enough to skew the numbers to favour the knockout T cells as was seen with IL-15 and IL-2 stimulated proliferation. Therefore, although there appears to be a skewing in favour of increased PSGL-1\textsuperscript{null} T cell proliferation compared to WT T cells, the differences in proliferation seen after ConA stimulation were not as extensive as the differences observed after homeostatic cytokine stimulation.
3.2.22 PSGL-1\textsuperscript{null} T cells have impaired survival \textit{in vitro}

In both the 5 week \textit{in vivo} proliferation assays (Figure 3.16) and in the \textit{in vitro} proliferation assays (Figure 3.18), we noted a decrease in viability of PSGL-1\textsuperscript{null} T cells compared to WT T cells. PSGL-1\textsuperscript{null} T cells lymph node cells placed in culture without cytokines were less viable than WT T cells in both CD4\textsuperscript{+} and CD8\textsuperscript{+} subsets (Figure 3.26A&B). T cells were initially mixed at a 1:1 ratio but after three days, the ratio of viable WT to PSGL-1\textsuperscript{null} T cells was approximately 70:30. When cultured with IL-7, the overall viability of all T cells increased. However, the ratio between WT and PSGL-1\textsuperscript{null} remained skewed in favour of the WT T cells. CCL19 has been shown to contribute to viability of T cells \textit{in vitro} and we have shown a clear interaction of PSGL-1 to CCL19 and CCL21 (Link et al., 2007). To determine if CCL19 and
**Figure 3.26**: WT T cells are more viable than PSGL-1\textsuperscript{null} T cells with homeostatic cytokines and chemokines. WT and PSGL-1\textsuperscript{null} lymph node cells were mixed at a 1:1 ratio, labelled with CFSE to ensure no division and placed in culture with medium alone, 10 ng/mL IL-7, 4 µg/mL CCL21, 1 µg/mL CCL19 or a combination thereof for three days. Viability was based on PI staining. Cells were later analyzed by flow cytometry and counting beads, and are presented based on fold difference to WT T cells with no cytokine control. Donor cells were identified using Thy1 and CD45 congenic markers. All experiments were done in triplicate and are representative of multiple experiments. Statistics are representative of the difference between WT and PSGL-1\textsuperscript{null}. ** p < 0.01 (Student's t-test). Error bars represent standard deviation.
CCL21 play a role in T cell viability in a PSGL-1-dependent manner, we added the chemokines to our *in vitro* viability assays. CCL19 and CCL21 did not show any contribution to T cell viability. When we combined IL-7 with CCL19 or CCL21 in cultures, there was no increase in T cell survival over IL-7 treatment alone, nor changes in the ratio of WT to PSGL-1<sup>null</sup> viable T cells. Therefore, it is evident that PSGL-1<sup>null</sup> T cells were not as viable as T cells expressing PSGL-1 regardless of cytokine or chemokine stimulation.

Piceatannol, a Syk and ZAP-70 inhibitor, has been linked to increased levels of apoptosis (Kim et al., 2009). We speculated that the inability of PSGL-1 to phosphorylate Syk may decrease the viability of T cells through a Syk dependent pathway. We added piceatannol at varying concentrations over 2 days in addition to IL-7. However, as seen in figure 3.27, adding piceatannol did not change the ratio of viable WT to PSGL-1<sup>null</sup> T cells. At higher concentrations, piceatannol killed all the cells. Therefore, we concluded that the decreased viability of T cells is not attributed to the signalling of PSGL-1 through Syk or ZAP-70 pathways.
Figure 3.27: Inhibition of Syk does not affect viability through PSGL-1. WT and PSGL-1null lymph node cells were mixed, labelled with CFSE to ensure no division and placed in culture with 10 ng/mL IL-7 and indicated amounts of piceatannol for two days. Viability was based on PI staining. Cultures were later analyzed by flow cytometry and counting beads were used to determine cell numbers. Donor cells were identified using Thy1 and CD45 congenic markers. All experiments were done in triplicate and are representative of multiple experiments. Error bars represent standard deviation. * p < 0.05, ** p < 0.01 (Student's t-test)
3.2.23 PSGL-1\textsuperscript{null} mice are capable of mounting a normal immune response but retain more memory T cells post infection than WT mice

Lastly, while this study has primarily focused on the role of PSGL-1 in T cell homeostasis and resting states, we were interested in knowing whether PSGL-1-deficiency and a decreased number of naïve T cells affects the ability of T cells to mount an immune response to foreign pathogens. Lymphocytic choriomeningitis virus (LCMV), a single-stranded RNA virus that is a member of the family Arenaviridae, is commonly used in mouse models to test viral pathogenesis and T cell activation. LCMV infection stimulates CD8+ cytotoxic T lymphocytes both in acute and chronic infections, is non-cytolytic and an acute infection can be cleared in 8 days (Oldstone et al., 1985). CD4+ T cells play a role with rapidly replicating and highly invasive strains of LCMV, such as LCMV-Traub but their contribution is negligible for less invasive strains such as LCMV-Armstrong (Ahmed et al., 1988; Battegay et al., 1994; Matloubian et al., 1994). It is also used for the study of memory T cells as memory T cells can be retrieved and enumerated from this model after 40 days.

We injected both WT and PSGL-1\textsuperscript{null} mice with 1x10\textsuperscript{5} pfu LCMV-Armstrong \textit{i.p.} and spleens were harvested on either Day 8 or Day 40 post-infection. After the clearance of the acute infection on day 8, both WT and PSGL-1\textsuperscript{null} mice showed comparable increases in their overall CD8+ T cell population as well as produced equal levels of LCMV specific CD8+ effector cells as measured by gp33 and np396 tetramers (Figure 3.28A,B&C). This suggests that despite the abnormalities in the T cell homeostasis in PSGL-1\textsuperscript{null} mice, their ability to mount an immune response was intact. After 40 days, CD8+ T cell numbers were reduced to approximately one fifth of those at Day 8 post infection and are comparable in WT and PSGL-1\textsuperscript{null} mice (Figure 3.28D). However, LCMV antigen specific CD8+ memory T cells, as measured by two different tetramers, showed a two to three fold increase in PSGL-1\textsuperscript{null} CD8+ memory T
cells compared to WT CD8+ memory T cells (Figure 3.28E&F). Representative tetramer staining shows clear LCMV reactive T cells (Figure 3.28G).
Therefore, PSGL-1\textsuperscript{null} mice were capable of mounting an efficient immune response despite initial decreased naïve T cell numbers in lymph nodes and peripheral blood. In contrast, numbers of CD8+ memory T cells 40 days post infection were augmented in PSGL-1\textsuperscript{null} mice compared to WT mice.
CHAPTER 4: DISCUSSION

4.1 Data summary

In this study we present five main conclusions. First, PSGL-1null mice have abnormal T cell profiles in the blood and lymph nodes. Second, PSGL-1 is required for efficient movement of T cells in and out of SLOs, and PSGL-1 function in SLO entry is dependent on chemokine binding. Third, PSGL-1null T cells spontaneously proliferate in vivo and proliferate more rapidly than WT T cells in response to homeostatic cytokines in vitro. Fourth, PSGL-1-deficient T cells are less viable than WT T cells both in vitro and in vivo. Fifth, PSGL-1null mice have an increase in memory T cells after immune challenge with LCMV.

4.2 PSGL-1null mice have subtle defects in their T cell subsets

PSGL-1 is a molecule that has been studied for almost twenty years and yet many aspects of its function are still being discovered. It was originally identified as a P-selectin ligand and up until recently has only been shown to play a role as a selectin ligand in recruitment of leukocytes to sites of inflammation. There have been two knockout mice made and studied, one by the lab of Bruce Furie (Yang et al., 1999) and the second by the lab of Rodger McEver (Xia et al., 2002). The PSGL-1null mouse produced by the Furie lab has been shown to have increased numbers of neutrophils in the peripheral blood yet lymphocyte numbers were comparable to WT mice (Yang et al., 1999). All of our studies were done using the Furie PSGL-1null mouse. The PSGL-1null mouse produced by the McEver lab was shown to have increased numbers of neutrophils, lymphocytes and eosinophils in the peripheral blood (Xia et al., 2002). The McEver PSGL-1null mouse has also been analyzed by the Consortium for Functional Glycomics as part of their general mouse phenotype screen. PSGL-1null mice were shown to have significantly reduced percentages of lymphocytes in the peripheral blood compared to WT mice but when absolute cell numbers were compared, there was no difference compared to WT controls (Consortium for Functional Glycomics, http://www.functionalglycomics.org/fg/). PSGL-1-
deficient mice have a large increase in numbers of neutrophils and eosinophils due to the inability of these cells to leave the blood stream and enter tissues, a phenotype also found in mice deficient in selectins or in mice deficient in glycosyltransferases needed for selectin ligand formation on PSGL-1 and other glycoproteins (Ellies et al., 1998; Maly et al., 1996; Mayadas et al., 1993). The difference in percentage of lymphocytes in PSGL-1null mice described by the Consortium of Functional Glycomics study is therefore a result of the skewing of an increase in neutrophils and eosinophils as opposed to a decrease in lymphocytes.

These studies reported either no difference or elevated numbers of lymphocytes in peripheral blood of PSGL-1null mice. However, none of these studies separated lymphocyte populations into B cells and T cells. Therefore, these studies overlooked the significant reduction in T cell numbers in the peripheral blood we report here as this was masked by the more abundant B cells that had a trend to be elevated. Our laboratory discovered PSGL-1 associated T cell lymphopenia only once we did a comprehensive analysis of B cell and T cell subsets which revealed the extent of the disruption in T cell homeostasis in PSGL-1null mice.

We have seen various differences between the T cells in WT mice compared to T cells in PSGL-1null mice. Nevertheless, what is remarkable is that lymph nodes, spleen and peripheral blood show different homeostatic disturbances and that lymphopenia cannot be generalized to the entire system. The spleen shows comparable numbers of CD4+ and CD8+ T cells and B cells in PSGL-1null mice and WT mice. This also applies to T cell subsets showing normal numbers of naïve T cells. In the lymph nodes there are comparable numbers of total T and B cells. However, there is an approximate 50% decrease in naïve T cells in PSGL-1null mice compared to WT mice. In peripheral blood, the differences between WT and PSGL-1null mice become even greater. Both CD4+ and CD8+ total T cell numbers are reduced by almost 50% in PSGL-1null mice. Upon analysis of T cell subsets, naïve T cell numbers have been reduced by up to 98% in PSGL-1null mice. Two reports from our laboratory provide possible mechanisms
that could explain the reduction of CD4+ and CD8+ T cell numbers in the blood of PSGL-1^nul
mice. One, PSGL-1^nul mice have been shown to have a reduced thymic output and therefore
mature thymocytes are slowed in entering the periphery (Gossens et al., 2009). Two, PSGL-1^nul
T cells have a reduced rate of entry into SLOs, especially lymph nodes (Veerman et al., 2007).
Since T cells that cannot enter lymph nodes have higher rates of apoptosis, this could also
contribute to T cell lymphopenia (Link et al., 2007). Nevertheless, these explanations for
reduced numbers of T cells in the blood do not explain why T cells in the blood are severely
affected and why lymph nodes and spleen are only mildly affected, or not affected all, especially
since T cells need to travel through the blood to enter SLOs.

The disappearance of PSGL-1^nul naïve T cells appears to be cell intrinsic based on our
bone marrow reconstitution studies. When we reconstituted IL-7R^nul mice with both WT and
PSGL-1^nul bone marrow cells, the resulting T cell profiles still show a decrease in the
percentage of PSGL-1^nul naïve T cells. However, T cells in the lymph nodes appear to be
relatively comparable between WT and PSGL-1^nul demonstrating that the discrepancy between
lymph node and peripheral blood compartments remain. Therefore, the T cell subset differences
between lymph nodes and blood is also cell intrinsic.

Levels of CD4^high memory T cells in the lymph nodes of PSGL-1^nul mice were normal.
This may be due to the fact that memory T cells are able to travel through the afferent lymph
into lymph nodes as well as via the HEVs. Although we did see a difference in PSGL-1-
dependent homing for memory T cells in our one hour in vivo homing assays, it is not clear
whether memory T cells entered lymph nodes via the blood through HEVs or through the
afferent lymph within that short time point. Memory T cells enter lymph nodes via the afferent
lymph by way of other tissues and therefore one hour may not have been a sufficient amount of
time to enter lymph nodes via that route.
It is surprising that there is not an increase in memory (phenotype) T cell numbers in PSGL-1\textsuperscript{null} mice as their T cells appear to be constantly proliferating causing upregulation of the memory marker CD44. Nevertheless, this may be explained by our data that show that PSGL-1\textsuperscript{null} T cells are less viable and thus disappear more rapidly from peripheral blood and SLOs. Therefore, slow, continuous proliferation of PSGL-1\textsuperscript{null} T cells may counter balance the increased rate of disappearance. Consistent with such a scenario is our observation that in aged PSGL-1\textsuperscript{null} mice (>one year), there is a trend for an increase in CD44\textsuperscript{high} memory T cell subsets in both CD4+ and CD8+ T cells compared to aged WT controls.

We also noticed discrepancies within the effector T cell subsets. L-selectin\textsuperscript{low}CD44\textsuperscript{lo} T cells in PSGL-1\textsuperscript{null} mice had a lower percentage of cells expressing CD69 and CD25 activation markers compared to WT, suggesting that the T cells we are classifying as effector cells had not lost L-selectin due to activation and therefore may instead include naïve or central memory phenotype T cells that have simply shed L-selectin.

### 4.3 PSGL-1 is needed for efficient homing of T cells to SLOs

We have shown that PSGL-1 is important for homing of naïve and memory T cells to SLOs, but not B cells. One hour after \textit{i.v.} injection of T cells into WT recipients, there were twice as many WT donor T cells present in the lymph nodes compared to PSGL-1\textsuperscript{null} donor T cells. We also saw a 30% reduction of PSGL-1\textsuperscript{null} T cells compared to WT in Peyer’s Patches and a 10% reduction in spleen. This reduction was not caused by the decrease in T cell viability of PSGL-1\textsuperscript{null} T cells since our T cell homing assays are analyzed after one hour, which is too soon to attribute to a decrease in viability. Both CD4+ and CD8+ were equally affected, as well as naïve T cells and memory T cells, although it should be noted that there were considerably less memory T cells that entered lymph nodes compared to naïve T cells. This may be due to the ability of memory T cells to enter lymph nodes through the afferent lymph as well as travel to other preferred sites such as the bone marrow (Di Rosa and Pabst, 2005; Mackay et al., 1990).
We did not see a PSGL-1-dependent effect on the homing of B cells. However this is most likely due to the fact that PSGL-1 is expressed at much lower levels on all B cells (Laszik et al., 1996).

Our discovery that PSGL-1 is needed for efficient homing of T cells to SLOs and for enhanced migration in *in vitro* chemotaxis transwell assays, through an interaction with CCL21 and CCL19, is novel and unexpected. Up until now, PSGL-1 function has always been associated with selectin binding. However, we found that this unique function of PSGL-1 is independent of interaction with any of the three selectins.

Our first indication that selectins may not be involved in PSGL-1-dependent homing of T cells to SLOs was that resting T cells do not express the necessary carbohydrate modifications needed to bind selectins (Vachino et al., 1995). Nevertheless, since the only documented function of PSGL-1 to date was as a selectin ligand, we speculated that there may be small amounts of selectins expressed on HEVs that had thus far been overlooked and were contributing to homing of T cells. While E-selectin and P-selectin are generally found expressed on blood vessels of inflamed tissues, there have been a number of studies that show these selectins to also be expressed in the HEVs during chronic inflammation (Takaeda et al., 2002) (Harakawa et al., 2007). Therefore, PSGL-1 may be interacting with E-selectin or P-selectin expressed on HEVs. Alternatively, L-selectin may be involved by binding to PSGL-1 through a secondary tethering mechanism. L-selectin, found on leukocytes, has been shown to bind to PSGL-1 on other leukocytes that are already bound to inflamed endothelium, thereby increasing T cell migration to areas of inflammation (Sperandio et al., 2003; Spertini et al., 1996). We speculated that L-selectin and PSGL-1 on adjacent cells in the HEVs may be interacting and creating a secondary tether and in turn increasing T cell migration to SLOs. To determine which selectin may be involved in PSGL-1-dependent homing, we did one hour *in vivo* competitive homing assays using P-selectin
null, E-selectin
null and C2GnT-1
null mice. C2GnT-1 is an essential glycosyltransferase needed for L-selectin (and P-selectin) binding to PSGL-1 and therefore,
C2GnT-1null T cells are not capable of supporting L-selectin/PSGL-1 interaction. Using a combination of C2GnT-1null and C2GnT-1null/PSGL-1null donor T cells in E-selectinnull recipients, we were able to eliminate the possibility of PSGL-1 interactions with any of the three selectins in one system. That PSGL-1null T cells remained at a disadvantage to enter lymph nodes in this experiment was an intriguing prospect.

PSGL-1 is involved in the first stages of the adhesion cascade mediating rolling and tethering in the recruitment of cells to sites of inflammation, through its low affinity interactions with selectins. However, since we determined that PSGL-1 did not interact with selectins in homing of T cells to SLOs, we shifted our focus to consider whether PSGL-1 might affect other stages of the adhesion cascade. An important second step in the lymphocyte homing cascade in HEVs involves chemokines, not only for attracting lymphocytes to the site of the HEV, but also for activating integrins and mediating the next stage of the adhesion cascade, firm adhesion. We first considered the possibility of chemokine involvement when a study from the Hirata group demonstrated that human PSGL-1 can bind CCL27, a chemokine that is expressed in the skin and attracts T cells during inflammation (Hirata et al., 2004). Through chemotaxis assays we were able to demonstrate that PSGL-1null T cells migrated less efficiently than WT T cells in response to CCL21 and CCL19. These findings allowed us to speculate that PSGL-1 homing to SLOs may involve specific interactions with homeostatic chemokines present in the HEVs, a new role for PSGL-1 that did not fall within the confines of the traditional role of PSGL-1 as a selectin ligand.

We considered the mechanism by which PSGL-1 was enhancing T cell migration to CCL21 and CCL19 and speculated that PSGL-1 may be involved in a pass-on mechanism. PSGL-1 is a large, negatively charged glycosylated protein that may capture CCL21 and CCL19 that is being released from HEVs and FRCs and prevent them from being dispersed in the bloodstream. Therefore, PSGL-1 may bind the chemokines and then pass them on to CCR7, their
corresponding receptor. When looking at the dose response of WT and PSGL-1null T cells toward CCL21, however, we can see that WT T cells always migrate better, even with saturating levels of chemokine. This observation would argue against such a pass on mechanism as there are abundant amounts of chemokine available for optimal migration. Instead, our data suggest that the increase in migration of T cells expressing PSGL-1 may be cell intrinsic.

An alternative explanation may be that PSGL-1 binding to CCL21 and CCL19 may alter their conformation so as to enhance the affinity of the chemokines for their receptor CCR7. While CCR7 is known to bind both CCL21 and CCL19 with high affinity in the absence of PSGL-1, we cannot exclude this possibility (Ott et al., 2006).

Another possibility for the mechanism of PSGL-1-dependent migration was PSGL-1 specific cell signalling in response to CCL21 and CCL19 interaction. Syk is a signalling molecule known to be downstream of PSGL-1, but it has not been described to be downstream of CCR7, and it has already been shown to play a role in chemotaxis (Schymeinsky et al., 2005; Urzainqui et al., 2002). ZAP-70, a homologue of Syk that is found in all T cells, has also been implicated to play a role in T cell chemotaxis (Dar and Knechtle, 2007; Ticchioni et al., 2002). Therefore we used the inhibitor piceatannol to determine if inhibition of Syk and ZAP-70 signalling affected PSGL-1-dependent T cell chemotaxis in response to CCL21 but found that it had no effect. Nevertheless, PSGL-1 has been shown to stimulate other signalling pathways and therefore may activate a separate signalling cascade that does not involve Syk. ERK, Rho, and Src kinases are all known to be downstream of PSGL-1, and are also linked to chemotaxis properties in other cell types. We therefore cannot exclude that PSGL-1-dependent homing is enhanced through another signalling pathway.

It is interesting that only migration to CCL21 and CCL19 is affected by the presence of PSGL-1 on T cells and not CXCL12, another important homeostatic chemokine. Chemokines
have been shown to bind other glycoproteins, such as glycosaminoglycans (GAGs) but were initially assumed to bind through nonspecific electrostatic interactions as chemokines are highly positively charged and glycosylated mucins such as GAGs and PSGL-1 are highly negatively charged. However, site-directed mutagenesis of CXCL12 showed that it binds to GAGs at a specific region, a region different from where its receptor CXCR4 binds so as not to compete for the same binding site (Amara et al., 1999). Therefore, it is not unlikely that PSGL-1 binds CCL21 and CCL19 in a site specific manner and not other homeostatic cytokines such as CXCL12. CCL21 and CCL19 have similar structures. However, CCL21 has an extended, highly basic 32 amino acid long region that CCL19 does not (Rey-Gallardo et al., 2010). This extended region has been implicated to bind GAGs which is why GAGs bind to CCL21 with a much higher affinity than CCL19 (Catusse et al., 2010). Since the PSGL-1 effect on migration occurs to a similar degree with both CCL21 and CCL19, it would suggest that PSGL-1 binding to CCL21 and CCL19 does not occur at the same site as GAG binding. Rather, it would suggest that PSGL-1 binds to CCL21 and CCL19 at a homologous site that also does not interfere with their interactions with CCR7. Since PSGL-1 presence enhances chemotaxis and homing, we would expect that PSGL-1 would not compete for the same binding regions of CCL21 and CCL19 as CCR7.

There are parallels that can be seen between PSGL-1-dependent T cell homing to SLOs and CCL21-deficient mice. To clarify, it is important to note that homing mechanisms between lymph nodes, Peyer’s patches and spleen have considerable differences. The adhesion molecules expressed on the HEVs of lymph nodes differ from those in Peyer’s patches, and the spleen lacks HEVs altogether. The spleen acts as a sieve in which lymphocytes are carried through the splenic artery and adhesion molecules on cells lining the marginal sinus catch the migrating lymphocytes. Nevertheless, CCL21 and CCL19 are involved in directing T cells out of the blood and into all three of these SLOs. In the plt/plt mouse which does not express CCL21
in SLOs, there are very few T cells present in lymph nodes and those that are present are mostly of a memory phenotype and therefore may be entering lymph nodes through afferent lymph and not through HEVs (Nakano et al., 1998). This indicates that CCL21 is required for T cell homing to lymph nodes. In Peyer’s patches of plt/plt mice there is a reduction in cellularity compared to WT mice but it is not as severe as the reduction in lymph nodes. In spleens of plt/plt mice, T cells were able to enter but they did not migrate to the T cell zones but were stuck in the vascular sinusoid of the red pulp. This suggests CCL21 is necessary to guide T cell movement within spleen but does not play as large of a role in the entry of T cells. The pattern of T cell cellularity and architecture in plt/plt mice follows a similar pattern as the PSGL-1-dependent homing we see in T cells. PSGL-1\textsuperscript{null} T cells show the greatest defect in T cell homing to lymph nodes where CCL21 is the most critical, whereas there is only a small decrease in T cell homing to spleen, where CCL21 is less critical for T cell entry.

PSGL-1 may be playing a dual, but related role in T cell migration through the body. First, it augments T cell entry into SLOs through a yet undetermined mechanism. Second, it may prevent T cells from re-entering lymph nodes after activation. T cell activation decorates the N-terminus of PSGL-1 with sLe\textsuperscript{x} and abrogates PSGL-1 enhanced chemotaxis toward CCL21 and CCL19. When we use activated C2GnT-1\textsuperscript{null} T cells to prevent sLe\textsuperscript{x} presentation upon activation, the PSGL-1-dependent migration in response to CCL21 is restored. Therefore, while PSGL-1 is important for resting T cells to enter lymph nodes, its altered glycosylation state after activation prevents it from binding CCL21 and CCL19 in HEVs and re-entering lymph nodes. It has been shown that L-selectin is shed from T cells after activation and therefore also prevents T cells from re-entering lymph nodes (Galkina et al., 2003). This study used non-sheddable L-selectin on T cells to determine that L-selectin shedding is needed for decreased LN re-entry after T cell activation. However, it also showed that activated T cells expressing non-sheddable L-selectin entered lymph nodes at a lower frequency than naïve T cells.
expressing non-sheddable L-selectin. This suggested that there was another mechanism independent of L-selectin shedding preventing T cells from re-entering lymph nodes after activation. We suggest that sLe$^\text{x}$ upregulation on PSGL-1 on activated T cells may constitute this second mechanism.

Our SLO exit studies have highlighted that PSGL-1 not only plays a role in SLO T cell entry, but it also affects the amount of time T cells spend in lymph nodes. CCL21 is also known to have a role in the direction of cell migration within lymph nodes (Kriehuber et al., 2001). The architecture of lymph nodes in PSGL-1$^{\text{null}}$ mice has been shown to be normal (Consortium of Functional Glycomics, http://www.functionalglycomics.org/fg/) suggesting that PSGL-1 is not critical for essential migration of cells within lymph nodes. However, it would be interesting to determine if PSGL-1$^{\text{null}}$ T cells move slower or with less direction in lymph nodes compared to WT T cells in response to CCL21. Therefore, the PSGL-1 effect on T cell residence time may be due to the inability of T cells to move within the lymph nodes and to move to areas that promote T cell exit. A possible way to measure T cell movement within lymph nodes would be to look at localization of WT and PSGL-1$^{\text{null}}$ T cells after lymph node entry using microscopy. Looking at various time points may show differences in migration patterns. Another more dynamic method would be to use 2-photon microscopy which allows for real time lymphocyte tracking in vivo (Bajenoff et al., 2006). This would allow evaluation of movement of WT and PSGL-1$^{\text{null}}$ T cells within the lymph nodes. Another possibility for increased residence time of PSGL-1$^{\text{null}}$ T cells in the lymph nodes is the inability of PSGL-1$^{\text{null}}$ T cells to react to cues that stimulate T cell exit such as S1P. S1P levels in peripheral blood have been shown to be moderately reduced in PSGL-1$^{\text{null}}$ mice and therefore may account for decrease in T cells exit (Gossens et al., 2009). However in our competitive egress assays, PSGL-1$^{\text{null}}$ T cells were in WT recipients and showed a decrease in lymph node exit rates compared to WT T cells, suggesting that the delay
in lymph node exit of PSGL-1null T cells is cell intrinsic and not due to altered S1P levels or other cues in the environment.

The defect in T cell migration of PSGL-1null T cells may contribute to the altered homeostatic T cell profiles we see in the PSGL-1null mouse. T cells that cannot enter the lymph nodes efficiently are deprived of necessary survival cytokines and therefore undergo apoptosis, suggesting a cause for the decrease in T cell numbers in the periphery. Nevertheless, it is surprising that despite this migrational defect, lymph node cellularity in PSGL-1null mice is normal. Other transgenic mice models that have reduced T cell migration to lymph nodes, such as L-selectinnull mice or FucTVIInull mice, also have a decrease in lymph node cellularity (Arbones et al., 1994; Maly et al., 1996). L-selectinnull mice also show an increase in circulating CD4+ T cells in the peripheral blood while CD8+ T cells are comparable to WT mice in young mice whereas our data showed PSGL-1null mice had a decrease in both CD4+ and CD8+ T cell numbers (Steeber et al., 1996). One commonality both PSGL-1null and L-selectinnull mice share, though, was that both mice develop splenomegaly upon aging, up to as much as twice as large and therefore had an overall larger number of lymphocytes than aged WT mice.

4.4 PSGL-1null T cells proliferate more than WT T cells

Homeostatic proliferation or, more accurately labelled lymphopenia-induced proliferation (LIP), is the process of T cell expansion when T cell counts are severely reduced in the body caused by, presumably, an excess of IL-7 available to stimulate proliferation. As we can see, PSGL-1null T cells are as capable as WT T cells at proliferating in response to lymphopenia in both an IL-7Rnull mouse model and an irradiated mouse model. However, when PSGL-1null T cells are unstimulated, they appeared to undergo spontaneous proliferation over a period of several weeks whereas WT T cells do not. We had anticipated that a PSGL-1null host may induce proliferation of donor T cells due to its marked lymphopenia but it is the PSGL-1null T cells themselves that seem to undergo proliferation irrespective of their environment.
Naïve T cells do not proliferate under normal circumstances and memory T cells have a slow division rate of once every 2-3 weeks (Boyman et al., 2009). Therefore, the discovery that PSGL-1null T cells were spontaneously dividing after 5 weeks was remarkable. As our data show that PSGL-1null T cells are delayed in exiting lymph nodes, we speculated that the increased time spent in lymph nodes may stimulate PSGL-1null T cells to proliferate in vivo under lymphoreplete conditions. Homeostatic proliferation has been shown to occur in lymph nodes where homeostatic cytokines are readily available to stimulate cell division (Schuster et al., 2009). We devised an in vivo experiment in which T cells were able to enter lymph nodes but were unable to exit lymph nodes. DOP is a chemical that disrupts S1P-dependent egress mechanisms causing T cells to become trapped in lymph nodes, as well as the thymus. By co-injecting both WT and PSGL-1null T cells into a single DOP treated recipient, both WT and PSGL-1null T cells would spend the same amount of time in the lymph nodes. After 5 weeks of DOP treatment, T cells trapped in the lymph nodes did indeed proliferate more than T cells that circulated freely. However, even when WT and PSGL-1null T cells were sequestered together in the lymph nodes, PSGL-1null T cells continued to proliferate at a higher rate than WT T cells. This clearly demonstrates that despite equal amounts of time spent in the lymph nodes exposed to the same microenvironment, PSGL-1null T cells still spontaneously proliferate in a cell intrinsic manner whereas WT T cells do not. However, we cannot completely exclude the possibility that WT and PSGL-1null T cells have different patterns of movement within the lymph nodes causing them to reside in different locations within the SLO. It is therefore feasible that PSGL-1null T cells may be exposed to different levels of cytokines than WT T cells if they are impaired in moving to certain areas. Nevertheless, PSGL-1null mice have normal SLO architecture (Consortium of Functional Glycomics, http://www.functionalglycomics.org/fg/) suggesting that PSGL-1null T cells may not be impaired in their movement around SLOs. Therefore, it more likely an increased sensitivity to cytokines present within the lymph nodes.
During our attempts to determine whether homeostatic chemokines may play a role in T cell proliferation, we were surprised to discover the extent to which T cells expand when exposed to cytokines in vitro without being activated or treated with any other stimulus. IL-15 caused massive proliferation in both PSGL-1null T cells as well as WT T cells at high doses, as did IL-2 to a lesser degree. This has been previously shown to occur with human T cells as well (Kanegane and Tosato, 1996). We were surprised that IL-15 and IL-2 could induce proliferation but IL-7 could not since IL-7 has repeatedly been shown to be the most essential factor in LIP as well as general T cell homeostasis (Surh and Sprent, 2008). In addition, when stimulated with IL-4, a cytokine that is known for its induction of Th2 CD4+ T cells upon activation, naïve CD8+ T cells also showed clear proliferation, another result we did not anticipate. However, the most remarkable result of all is that PSGL-1null CD8+ T cells responded quicker to these cytokines and proliferated more rapidly than WT T cells.

It has been shown by others that PSGL-1null T cells proliferate more rapidly than WT T cells during in vitro activation (Matsumoto et al., 2009). Our data with ConA blasts also showed a mild increase in proliferation of PSGL-1null T cells compared to WT T cells. This could indicate that PSGL-1null T cells are hyper-proliferative to any stimuli. There was no difference in the proliferation of WT and PSGL-1null T cells in IL-7Rnull hosts however there was a trend for an increase in proliferation of PSGL-1null CD8+ T cells in sublethally irradiated hosts. This may then suggest that T cells that PSGL-1 negatively regulates proliferation both in inflammatory settings as well as homeostatic proliferative settings.

Memory T cells express higher levels of IL-15Rα and IL-2Rα than naïve T cells and respond quicker to IL-15 and IL-2. While our data show that PSGL-1null mice do not have increased absolute numbers of memory T cells compared to WT mice, there is a general trend toward an increase in the percentage of memory T cells in lymph nodes. Therefore, we needed to exclude that the increase in PSGL-1null T cell proliferation was due to larger proportion of
memory T cells in the lymph node cell preparation of PSGL-1null mice. Sorted PSGL-1null CD8+ T cells maintained their proliferative advantage over sorted WT T cells, both in naïve and memory T cell subsets, excluding such an artefact. We did confirm, however, that memory T cells proliferated at a faster rate than naïve T cells in response to homeostatic cytokines, as previously reported (Zhang et al., 1998).

Both naïve T cell viability and homeostatic proliferation in vivo is dependent on TCR stimulation by MHC (Ernst et al., 1999; Tanchot et al., 1997). Depletion of APCs from T cell cultures, however, did not impede proliferation in response to IL-15 and thereby demonstrates that MHC on APCs is not required for T cell proliferation in response to homeostatic cytokines in vitro. However, we cannot exclude the possibility that the CD8+ T cells may stimulate each other via an MHC class I:TCR interaction as documented in other studies (Cho et al., 2007). It is difficult to interpret these data since MHC has been shown to be necessary for homeostatic proliferation in vivo for naïve T cells, but not memory T cells (Surh and Sprent, 2008).

To exclude the possibility that differences in proliferation in response to homeostatic cytokines was caused by differences in cytokine receptor expression, we measured levels of γc family cytokine receptors on both WT and PSGL-1null T cells. Our data showed that there are no differences between T cells from lymph nodes of WT and PSGL-1null mice. We tested γc, IL-2β, IL-2α and IL-15α. After stimulation with cytokines, cell surface expression levels of the receptors rose in T cells that had proliferated but there were no differences between T cells with or without PSGL-1. There was a small but significant increase in IL-2Rβ expression on PSGL-1null T cells compared to WT T cells stimulated in culture with IL-15, but not on T cells taken ex vivo, making it difficult to interpret whether these data were relevant. We do not, however, believe that this small increase in IL-2Rβ expression on PSGL-1null T cells was the underlying cause for increased proliferation as PSGL-1null T cells also showed increased proliferation in response to IL-4, which does not bind to IL-2Rβ.
We tested many different cytokines including IL-2, IL-4, IL-7, IL-15, IL-12, IL-6 and even the chemokines CCL21 and CCL19 for their capacity to stimulate proliferation in resting lymph node cells. However, CD8+ T cells alone responded to IL-2, IL-4 and IL-15, three members of the γc family of cytokines. It can, however, not be generalized to all γc family members since CD8+ T cells did not proliferate in response to IL-7.

CD8+ T cells do not express IL-2Rα and yet IL-2 stimulated both WT and PSGL-1null CD8+ T cells to proliferate. IL-2 has been shown to bind directly to IL-2Rβ with intermediate affinity and cause downstream signalling which may provide an explanation as to why CD8+ T cells still proliferate despite a lack of IL-2Rα expression on CD8+ T cells (Gonzalez-Garcia et al., 1997). IL-15 stimulated T cells proliferated considerably more than IL-2. This may be attributed to the fact that IL-15 binds to IL-15Rα with a much higher affinity than IL-2Rα, almost three fold more. However, as IL-2Rα is not expressed on CD8+ T cells, it is much more likely that IL-2 is signalling through the IL2R beta chain which has a lower affinity for cytokine binding when it is not coupled to its alpha chain. IL-4 is primarily known for its effects on activated T cells but IL-4 has been noted to be involved in homeostatic proliferation. After IL-4 injection into Bl/6 mice, very little proliferation was seen. However, when IL-4 is complexed with IL-4 mAb, almost all CD8+ T cells were stimulated to proliferate (Boyman et al., 2006b). This, however, was also shown to occur with IL-7/IL-7 mAb complexes as well whereas we did not see proliferation in response to IL-7 in our system.

One possibility for the hyper-proliferative response of PSGL-1null T cells to cytokines could be the fact that they are already proliferating in vivo and therefore may require less time to respond to cytokines in vitro. Indeed, priming OT-I transgenic T cells in vivo by injection into irradiated hosts for 3-15 hours was sufficient to make the T cells proliferate in response to IL-7, IL-4 and IL-15 in vitro (Tan et al., 2001). CD8+ T cells in a PSGL-1null mouse may thus be simply primed to proliferate in vivo and therefore respond better to homeostatic cytokines in vitro.
The study mentioned above used OT-I transgenic T cells which are often used in homeostatic proliferation studies because it is one of the few transgenic mouse models that is capable of undergoing homeostatic proliferation. It has a high affinity TCR which is very easily stimulated by MHC, a necessary component of LIP. However, OT-I T cells can also be stimulated by endogenous ligands in a WT mouse to spontaneously proliferate (Kieper et al., 2004). Therefore, when our lab injected OT-I and OT-I/PSGL-1null T cells in WT recipients we saw general proliferation in both donor types and it was difficult to distinguish whether there were differences when PSGL-1 was present or not. We considered the idea that since OT-I T cells express a high affinity transgenic TCR and proliferate more, perhaps PSGL-1null mice also have a bias toward high affinity TCRs due to slow proliferation over time within the lymphopenic mouse, a process that favours T cells expressing high affinity receptors. However, studies done previously in our lab looking at T cell repertoire diversity in PSGL-1null mice suggested no difference compared to WT mice.

Another possibility for the increase in cytokine responsiveness of PSGL-1null T cells could be the stimulation of further cytokine release by these cells, thereby increasing the levels of cytokine available and stimulating more proliferation. This however, is unlikely since WT and PSGL-1null T cells are co-cultured in vitro and therefore are exposed to the same levels of cytokine, irrespective of the amounts being produced. The differences seen between WT and PSGL-1null T cells while co-cultured would then suggest that PSGL-1null T cells are hyper-proliferative for reasons that are independent of external stimuli.

Syk has been linked to proliferation and has been shown to associate with the IL-2, IL-4 and IL-15 receptor complexes (Ennaciri and Girard, 2009; Minami et al., 1995; Ratthe and Girard, 2004; Zhou et al., 2000). Therefore, we speculated that PSGL-1 may modulate Syk signalling of these receptors. When we added the Syk inhibitor piceatannol, we observed that overall proliferation was reduced yet PSGL-1null T cells still had an advantage over WT T cells.
suggesting that PSGL-1 does not affect Syk signalling downstream of cytokine receptors. ZAP-70 is a homologue of Syk that is downstream of the TCR on T cells that is also inhibited by piceatannol. Since piceatannol had an inhibitory effect on the proliferation of T cells in response to IL-15 it would suggest either Syk or ZAP-70 may play a role in proliferation \textit{in vitro}.

PSGL-1\textsuperscript{null} mice have a trend for an increase in \textit{CD44}\textsuperscript{high} \textit{L-selectin}\textsuperscript{low} effector memory T cells in the peripheral blood and lymph nodes compared to WT mice. In our \textit{in vitro} studies we showed that T cells dividing in response to IL-15 upregulate CD44 and downregulate L-selectin. Therefore, the increase in this subset of T cells in PSGL-1\textsuperscript{null} mice may be due to the slow proliferation of \textit{CD44}\textsuperscript{low} \textit{L-selectin}\textsuperscript{high} naïve T cells converting into a \textit{CD44}\textsuperscript{high} \textit{L-selectin}\textsuperscript{low} effector memory phenotype subset.

We observed that the effector subset in PSGL-1\textsuperscript{null} lymph node T cells showed a reduced percentage of \textit{CD69}\textsuperscript{+} and \textit{CD25}\textsuperscript{+} T cells. However, we see \textit{CD69} and \textit{CD25} upregulation on memory phenotype T cells during our \textit{in vitro} proliferation assays. Therefore, if spontaneous proliferation in response to homeostatic cytokines was occurring in PSGL-1\textsuperscript{null} mice, we would expect equivalent, or even increased percentages of \textit{CD69}\textsuperscript{+} and \textit{CD25}\textsuperscript{+} expressing T cells in these mice. We cannot exclude however, that \textit{CD69} and \textit{CD25} expression on dividing PSGL-1\textsuperscript{null} T cells \textit{in vivo} may be different than \textit{CD69} and \textit{CD25} expression on dividing T cells after cytokine stimulation \textit{in vitro}. While the upregulation of these early activation markers is not typically observed in homeostatic expansion, there have been studies that show proliferating T cells in LIP models can upregulate \textit{CD69} and \textit{CD25}, particularly in genetically lymphopenic hosts, such as the Rag-deficient mouse (Jameson, 2002). It has also not been determined whether there are differences in the levels of expression of \textit{CD69} and \textit{CD25} expression between proliferating activated T cells and memory phenotype T cells, or if \textit{CD69} and \textit{CD25} are downregulated at a different rate between these two cell types.
4.5 PSGL-1\textsuperscript{null} T cells are less viable than WT T cells

The decreased viability of PSGL-1\textsuperscript{null} T cells \textit{in vitro} adds another dimension to the increase in proliferation of PSGL-1\textsuperscript{null} T cells seen \textit{in vitro}. After two days in culture, when the CD8+ T cells had not yet noticeably begun to divide, there were approximately two to three fold more WT T cells to PSGL-1\textsuperscript{null} T cells despite having started at a 1:1 ratio. After four days in culture, the ratio of PSGL-1\textsuperscript{null} T cells to WT T cells skewed in favour of the former. This would imply that the rate of PSGL-1\textsuperscript{null} T cell proliferation was even higher than what our data suggested because they started at a disadvantage, as more PSGL-1\textsuperscript{null} T cells had died in the early culture period.

It could be proposed that PSGL-1\textsuperscript{null} lymph node cells \textit{in vitro} are losing a particular subset of cells and that the remaining viable subset is more sensitive to IL-15 stimulation, accounting for the increase in proliferation in PSGL-1\textsuperscript{null} cells. However, both viable naïve and memory T cells from both WT and PSGL-1\textsuperscript{null} mice were found in cultures after 4 days in media alone. Therefore, there is no bias in viability of a particular memory or naïve subset of CD8+ T cell that explain the decrease in viability of PSGL-1\textsuperscript{null} T cells. We cannot, however, completely exclude the possibility of another CD8+ cell subset that may be skewing the data somehow. We did note that sorted CD8+ T cells placed in culture without APCs or cytokines did not survive at all whereas a small population of T cells generally persisted when whole lymph node preparations were cultured, suggesting that another cell type present in whole lymph node preparations may help some T cells remain viable.

Under competitive \textit{in vivo} conditions, less PSGL-1\textsuperscript{null} T cells were retrieved than WT T cells. Within days, the ratio of T cells skewed in favour of T cells expressing PSGL-1 both in blood and lymph nodes. Nevertheless after 5 weeks there were still PSGL-1\textsuperscript{null} T cells present suggesting that the few remaining T cells are dividing, increasing in numbers and achieving an equilibrium. When we measured the half-life of thymocytes instead of lymphocytes, we saw the
ratio of WT over PSGL-1<sup>null</sup> T cells grow even more. The increased loss of PSGL-1<sup>null</sup> thymocytes from the blood compared to PSGL-1<sup>null</sup> lymphocytes may be due to the fact that these cells have not yet been in the periphery, and have not yet been selected based on optimal survival. In a PSGL-1<sup>null</sup> mouse, if T cells are unable to enter lymph nodes efficiently and therefore they are deprived of homeostatic cytokines for longer periods than T cells in a WT mouse, it may induce a “survival of the fittest” scenario. T cells that are less robust, less capable of entering lymph nodes or less responsive to cytokine stimulation may disappear quicker, leaving only T cells have an increased advantage for survival. Perhaps T cells in a PSGL-1<sup>null</sup> mouse that do manage to enter lymph nodes have increased sensitivity to homeostatic and proliferative cytokines or upregulated cytokine receptors due to increased time spent in lymph nodes caused by their defect in egress. Therefore, when we use T cells from the lymph nodes of PSGL-1<sup>null</sup> mice they already may be pre-selected to survive the conditions of a PSGL-1<sup>null</sup> mouse.

However, while this theory may support the idea of PSGL-1<sup>null</sup> T cells being pro-proliferative, it does not support the decrease in viability of PSGL-1<sup>null</sup> T cells. If T cells are being selected based on the fittest then we would expect that PSGL-1<sup>null</sup> T cells would actually survive better than WT T cells <em>in vitro</em> and <em>in vivo</em>. Instead, our data suggested that T cells in a PSGL-1<sup>null</sup> mouse are pro-apoptotic. This is logical for T cells in the peripheral blood because they are deprived of homeostatic cytokines for longer periods of time because they cannot enter lymph nodes efficiently, but if they have already made their way into the lymph node, we would expect that they would have received pro-survival signals within the lymph nodes and therefore when cultured <em>in vitro</em>, WT and PSGL-1<sup>null</sup> T cells would be comparably viable. It is possible that PSGL-1<sup>null</sup> lymph nodes cannot provide sufficient survival signals but if that were the case, we would not expect the lymph nodes of PSGL-1<sup>null</sup> mice to be normal in size, but would expect them to be smaller with reduced numbers of lymphocytes. In reality, they are often visually
larger than lymph nodes from a WT mouse. If T cells are proliferating in response to homeostatic cytokine stimulation there would have to be an excess of cytokines available within the lymph nodes. However, due to their normal cellularity, we would expect that levels of homeostatic cytokines would be too low to stimulate proliferation based on the model that homeostatic proliferation occurs when cell numbers are diminished and IL-7 and other cytokine levels are increased (Surh and Sprent, 2008). In addition, data from our 5 week competitive proliferation assays in WT hosts demonstrated an increase in proliferation of donor PSGL-1<sup>null</sup> T cells suggesting the PSGL-1 effect to be cell intrinsic and not due to different cytokine levels in the PSGL-1<sup>null</sup> mouse.

Another aspect to consider is if T cells were indeed continuously proliferating throughout the lifespan of the mouse, we would expect to see some signs of autoimmunity, which is associated with constant homeostatic proliferation. When our laboratory looked for signs of autoimmunity in aged mice (one year or older), however, there were no obvious indications. The only remarkable difference between aged WT and PSGL-1<sup>null</sup> mice was the size of the spleen which was twice as large in the PSGL-1<sup>null</sup> mouse.

4.6 PSGL-1<sup>null</sup> mice retain a larger number of memory T cells after immune challenge

We showed that PSGL-1<sup>null</sup> mice are capable of mounting a normal immune response against LCMV challenge despite the homeostatic defects we see in the T cell compartment. After 8 days, there were comparable numbers of LCMV specific CD8<sup>+</sup> T cells in WT and PSGL-1<sup>null</sup> mice as well as total numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen. This suggested the reduction in naïve T cell numbers lymph nodes and peripheral blood of PSGL-1<sup>null</sup> mice did not affect its ability to respond to LCMV challenge. This was consistent with a previous study where it was shown that lymphopenic mice with only 20% the numbers of CD8<sup>+</sup> T cells in spleen were capable of mounting a similar immune response to LCMV challenge as WT mice (Lin et al., 2008).
Interestingly, while PSGL-1\textsuperscript{null} naïve T cells mount a normal immune response to LCMV challenge, LCMV-specific memory T cells in PSGL-1\textsuperscript{null} mice after 40 days are 2.3 to 2.7 fold higher than in WT mice. We propose various possibilities for this.

We speculated that memory T cells expressing higher levels of cytokine receptor on their surface may proliferate more and increase cell numbers if lymph nodes in a PSGL-1\textsuperscript{null} mouse are indeed producing higher levels of homeostatic cytokines causing spontaneous proliferation of T cells. Nevertheless, the possibility that PSGL-1\textsuperscript{null} mice produce larger amounts of cytokine is unlikely and does not satisfactorily explain why PSGL-1\textsuperscript{null} T cells proliferate better than WT T cells in a WT host under non-inflammatory conditions.

A recent study showed that mice with impaired lymph node entry also showed an increase in memory T cells post LCMV infection (Harp et al., 2010). FucT-IV and –VII-deficient mice cannot synthesize selectin ligands in the HEVs and therefore L-selectin mediated lymph node entry is impaired and cellularity in lymph nodes is severely reduced. While overall numbers of LCMV responding T cells were reduced due to inability to enter lymph nodes, after 60 days there were equal amounts of LCMV-specific memory T cells in the knockout mice compared to WT, and therefore the proportion of memory T cells was much higher in the knockout mice. This group attributed this increase in memory T cell frequency to a number of factors. First, memory T cells prefer to enter lymph nodes through the afferent lymph which is selectin ligand independent as opposed to the HEVs which relies on L-selectin mediated rolling. Second, T cell egress is reduced when T cell numbers in the lymph nodes are diminished. Third, LIP, caused by lymphocyte depleted lymph nodes, favours memory T cells as they express high levels of homeostatic cytokine receptors such as IL-15Rα. Since lymph nodes in a PSGL-1\textsuperscript{null} mouse are not depleted and we have shown that memory T cells are also affected by PSGL-1-dependent homing to lymph nodes, these theories do not explain why PSGL-1\textsuperscript{null} mice have increased numbers of LCMV-specific memory T cells after 40 days. Nevertheless, we do
see spontaneous T cell proliferation as well as reduced exit under normal circumstances and so while we cannot attribute it to decreased lymph node cellularity, it may explain why bone fide memory T cells are enriched in PSGL-1\textsuperscript{null} mice after LCMV infection.

In another study using LCMV challenge, a similar increase in memory T cells was found in mice having been reconstituted by LIP. T cell knockout mice were reconstituted with WT splenocytes over 5 weeks and then challenged with LCMV (Lin et al., 2008). Reconstituted mice initially had 20% of the number of T cells compared to WT mice before infection yet they mounted an immune response with comparable levels of responding CD8+ T cells after 8 days. They also showed that after LCMV clearance, CD8+ T cell numbers in reconstituted mice were reduced to levels comparable to WT mice, but not to initial lymphopenic levels seen before LCMV infection. LCMV-specific CD8+ memory T cells after 6 months were 4-6 fold higher levels in reconstituted mice than in WT mice. The authors suggested the cause of the increased memory T cell numbers in reconstituted mice was due to LCMV specific memory cells filling the depleted T cell compartment as infection resolves. Since the T cell compartment was not full initially, stimulating an immune response and thereby releasing cytokines such as IL-2 and IL-15 may encourage T cells and memory T cells in particular to expand. This reasoning matches that of the FucT-IV and –VII knockout mice in that reduced T cell compartments may favour memory T cell expansion. Therefore, the general proliferation we see in PSGL-1\textsuperscript{null} T cells, the reduction in egress times, the increase in cytokine response and the increase in cytokines released during LCMV infection may explain why PSGL-1\textsuperscript{null} mice have an increased number of CD8+ memory T cells after LCMV challenge. Nevertheless, this theory is very speculative and would need to be confirmed with further experimentation. One possible way to test this would be to test the LCMV model in IL-15-, IL-2-, and/or IL-4-deficient mice crossed with PSGL-1-deficiency to determine if one of these three cytokines stimulate the increase in memory T cells in a PSGL-1-deficient environment.
4.7 Data summary

A graphical representation of the major conclusions of the data can be found in Figure 4.1. PSGL-1\textsuperscript{null} mice have abnormal numbers of T cells, in particular, a decrease of total T cells in the blood and a decrease of naïve T cells in the blood and lymph nodes. Also, PSGL-1\textsuperscript{null} T cells are less efficient in entering and exiting SLOs. SLO entry is regulated by the interaction of PSGL-1 with CCL21 and CCL19. PSGL-1\textsuperscript{null} CD8+ T cells spontaneously proliferate \textit{in vivo} and when stimulated with IL-15, IL-2 or IL-4, proliferate more rapidly than WT CD8+ T cells \textit{in vitro}. However, PSGL-1\textsuperscript{null} T cells are less viable both \textit{in vivo} and \textit{in vitro}. Despite these disturbances in T cells homeostasis, PSGL-1\textsuperscript{null} T cells can mount an efficient immune response to LCMV which is comparable to WT T cells. However, loss of PSGL-1 is associated with an increased memory T cell response after immune challenge.

\textbf{Figure 4.1: Summary}. PSGL-1\textsuperscript{null} mice have reduced T cells in the blood, and reduced naïve T cells in the blood and lymph nodes. PSGL-1\textsuperscript{null} T cells cannot enter nor exit lymph nodes efficiently. They also have reduced viability in the blood, lymph nodes and \textit{in vitro}. PSGL-1\textsuperscript{null} T cells proliferate quicker in response to homeostatic cytokine stimulation \textit{in vitro}, which may contribute to the increase in memory T cells in PSGL-1\textsuperscript{null} mice 40 days after immune challenge with LCMV.
4.8 Clinical implications

Homeostatic proliferation has been explored for its potential as a therapy in situations of immune suppression such as chemotherapy treated patients, bone marrow transplant recipients and HIV-infected individuals. PSGL-1 has been used as an antibody target for immune suppressive therapies in autoimmune diseases with little success. However, these novel roles of PSGL-1 in T cell homing and T cell proliferation may offer an alternative target. While we have shown that PSGL-1 deficiency promotes T cell expansion, there is no overexpansion in vivo and no indication of autoimmune disease. Therefore, PSGL-1, or the mechanism by which PSGL-1 enhances T cell proliferation, may provide a novel way to induce therapeutic homeostatic proliferation of T cells without the side effects of uncontrolled self-reactive T cell proliferation that is often seen with IL-7 and/or IL-2 cytokine based therapies.

4.9 Future directions

This study has explored and discovered a number of new roles for PSGL-1, particularly under homeostatic settings. We have shown that PSGL-1 is important for T cell migration into lymph nodes in a CCL21 and CCL19 dependent manner. We also have shown that PSGL-1null T cells are retained in lymph nodes but whether this is due to defective migration in the lymph nodes or an inability to receive necessary cues for lymph node exit is still uncertain. It would be interesting to determine how PSGL-1 interacts with CCL21/19 to affect T cell homing and whether those interactions also play a role in movement of T cells within the lymph nodes where CCL21 and CCL19 are also known to be important. Monitoring the movement of PSGL-1null T cells within the lymph nodes using microscopy may provide some insight as to what is occurring there. While we have also shown that viability of T cells is diminished when PSGL-1 is not present and that PSGL-1null T cells are hyper-proliferative both in vitro and in vivo, it is still not clear whether the effects we see are cell intrinsic or whether they are a compensatory mechanism caused by their deficiencies in homing. Determining the underlying mechanism
behind the decrease in viability and increased proliferation, two very different processes, would be necessary to determine if PSGL-1 is playing a direct role either through signalling or through cytokine interaction, or whether T cells from a PSGL-1\textsuperscript{null} mouse have a homing defect that is indirectly affecting their behaviour in other areas. Future studies may focus on other signalling molecules downstream of both PSGL-1 and homeostatic cytokine and chemokine receptors. Alternatively, HSCs can be differentiated into functional DP and SP thymocytes \textit{in vitro} using OP9-DL1 cells, a bone marrow stromal cell line expressing Notch ligand Delta-like-1. Studying T cells differentiated within this system may address the question whether PSGL-1\textsuperscript{null} T cells have an altered viability and proliferation profile due to an intrinsic defect or whether the differences we see are caused as a reaction to migrational defects. T cells produced in this \textit{in vitro} system would bypass migrational dependency.
REFERENCES


