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Adhesion Molecule Regulation of Regulatory T Cell Migration

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by

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ABSTRACT
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Regulatory T (Treg) cells mediate tumor immune evasion by suppressing anti-tumor effector T cell responses in peripheral lymphoid tissues and within the tumor. While elevated Treg cell numbers have been shown to correlate with increased tumor growth, mechanisms that regulate their distribution within secondary lymphoid tissue and tumor tissue are not well understood. L-selectin, an adhesion molecule constitutively expressed on all classes of leukocytes, functions early in the adhesion cascade and regulates the migration of lymphocytes to lymph nodes through high endothelial venules. In addition, L-selectin can mediate migration of lymphocytes to sites of inflammation by binding to ligands present on inflamed endothelium. Treg cells express high levels of L-selectin and require L-selectin for entry into resting lymph nodes. However, the role of L-selectin in regulating Treg cell distribution and migration into tumors and lymph nodes during chronic inflammation, such as cancer, has not been examined. Therefore, we investigated the role of L-selectin in regulating Treg cell rolling and adhesion to an endothelial cell monolayer *in vitro* as well as the distribution and migratory patterns of Treg cells using the murine 4T1 breast cancer model. Importantly, $\alpha_4\beta_7$ integrin/VCAM-1 interactions

were found to significantly contribute to Treg cell adhesion to endothelial cell monolayers under shear stress only in the presence of L-selectin function. *In vivo*, Treg cell populations preferentially accumulated in tumors and tumor-draining lymph nodes during progression of disease, and increased at higher rates than conventional $CD4^+$ T cell populations as tumors progressed. Furthermore, Treg cells preferentially migrated to tumors and tumor-draining lymph nodes in a L-selectin-dependent manner, thereby promoting immune suppressive environments. These studies provide further insight into the mechanisms of L-selectin function in regulating Treg cell distribution during chronic inflammation such as cancer, and may lead to a better understanding of tumor immune evasion and provide new targets for immunotherapeutic strategies.

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3.	926-FtVII/VCAM-1	α1,3-fucosyltransferase and VCAM-1transfected EA.hy926 cell line
4.	926-VCAM-1	VCAM-1transfected EA.hy926 cell line
5.	APC	antigen presenting cell
7.	CCR	CC-chemokine receptor
8.	CLA-1	cutaneous lymphocyte antigen-1
9.	CNS	conserved non-coding sequences
10.	CTLA-4	cytotoxic T-lymphocyte antigen-4
11	DC	dendritic cells
12.	dLN	tumor-draining lymph node
13.	EGFP	enhanced green fluorescent protein
14	ELC	EB11 ligand chemokine
15.	Foxp3	forkhead box p3
16.	FBS	Fetal bovine serum
17.	FITC	Fluorescein isothiocyanate
18.	$Foxp3^{EGFP}$	Balb/c Foxp3EGFP reporter
19.	$Foxp3^{EGFP}$/L-$selectin^{-/-}$	Balb/c Foxp3EGFP L-selectin-deficient reporter
20.	FtVII	fucosyltransferase-VII
21.	G-CSF	granulocyte colony stimulating factor
22.	GM-CSF	granulocyte-macrophage colony stimulating factor
23.	G-MDSC	granulocytic myeloid derived suppressor cell
24.	HECA-452	human endothelial cell antigen-452
25.	HEV	high endothelial venule
26.	ICAM-1	intercellular cell adhesion molecule-1
27.	IDO	indoleamine 2,3-dioxygenase
28.	IFN-γ	interferon-γ
29.	Ig	immunoglobulin
30.	IL	interleukin
31.	IL-2R	IL-2 receptor
32.	iTr35	IL-35 producing Treg cell
33.	iTreg cell	Induced Treg cell
34.	LFA-1	lymphocyte function associated antigen-1
35.	L-$selectin^{-/-}$	L-selectin-deficient
36.	mAb	monoclonal antibody
37.	MAdCAM-1	mucosal addressin cell adhesion molecule-1
38.	MDSC	myeloid derived suppressor cell

39.	MHC	major histocompatibility complex
40.	MLN	mesenteric lymph node
41.	M-MDSC	monocytic myeloid derived suppressor cell
42.	ndLN	non-tumor-draining lymph node
43.	NFAT	nuclear factor of activated T cell
44.	NK	natural killer
45.	NKT	natural killer T cell
46.	nTreg	natural Treg cell
47.	PE	phycoerythrin
48.	PECAM-1	platelet-endothelial cell adhesion molecule-1
49.	PLN	peripheral lymph node
50.	PNAd	peripheral node addressin
51.	PSGL-1	P-selectin glycoprotein ligand-1
52.	SLC	secondary lymphoid-tissue chemokine
53.	STAT5	signal transducer and activator of transcription-5
54.	TCR	T cell receptor
55.	Tfh	follicular helper T cells
56.	TGF-β	transforming-growth factor-β
57.	Th1	helper type 1 T cell
58.	Th17	interleukin-17 producing T helper
59.	Th2	helper type 2 T cell
60.	Th22	IL-22 producing T helper cell
61.	Th9	IL-9 producing T helper cell
62.	TIL	tumor-infiltrating lymphocyte
63.	TK-1$^{L\text{-}sel}$	L-selectin-transfected TK-1 T cell
64.	TNF-α	tumor necrosis factor-α
65.	Tr1	T regulatory 1 cells
66.	Treg	regulatory T cell
67.	VCAM-1	vascular cell adhesion molecule-1
68.	VEGF	vascular endothelial growth factor
69.	VLA-4	very late antigen-4
70.	WT	wild type

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CHAPTER 1

GENERAL INTRODUCTION

I. Introduction

The vertebrate immune system is a dynamic system comprised of white blood cells, or leukocytes, that circulate throughout the body. Recirculation of lymphocytes, one type of leukocyte, through secondary lymphoid tissue is critical for surveillance of foreign pathogens or malignant cells, and enables the immune system to mount a rapid immune response. During inflammation, leukocytes actively migrate out of the blood stream in response to tissue damage or inflammatory mediators and enter tissues to clear pathogens or eliminate tumor cells. Recirculation and migration are regulated by adhesion molecules present on the leukocytes that bind to complementary ligands present on either endothelium or other leukocytes. The coordination of leukocyte adhesion molecule expression and vascular endothelial ligand expression regulates the recruitment of specific leukocytes, such as subsets of lymphocytes, into lymphoid or peripheral tissues to clear target pathogens or cells. Once the target has been cleared, suppressive leukocytes function to dampen immune responses and return the body to homeostasis. However, during chronic inflammation, such as cancer, suppressive cells, such as regulatory T (Treg) cells, can dampen anti-tumor responses, thereby providing protection to the growing tumor cells. Little is known about adhesion molecule regulation of Treg cell recirculation or migration during chronic inflammatory conditions. Thus, determining adhesion molecule regulation of Treg cell migration is essential for understanding immune responses to tumors and developing therapeutic strategies to boost anti-tumor immune responses and enhance currently available immunotherapies.

II. Helper T cells

Lymphocytes are generated from progenitor stem cells within the bone marrow, and remain in the bone marrow for maturation (B lymphocytes) or migrate to the thymus to mature (T lymphocytes). Upon maturation, lymphocytes recirculate within the blood stream and become enriched in secondary lymphoid tissues, such as the spleen, lymph nodes, and Peyer's patches. Lymphocytes consist of several subsets, namely helper T cells, cytotoxic T cells, and B cells, based upon function and surface molecule expression. Adaptive immune responses are mediated by individual, highly-specific lymphocyte recognition of unique peptides, or antigens, present on foreign pathogens or malignant cells. Once a lymphocyte has encountered its specific antigen, it becomes activated and undergoes proliferation to generate a cohort of cells to clear the infection or tumor. Once the target has been cleared, the expanded population of lymphocytes undergo apoptosis, but a small subset remain as memory cells and provide long-lasting immunologic memory.

One subset of T lymphocytes, T helper cells, is characterized by expression of the CD4 co-receptor and termed $CD4^+$ T cells. $CD4^+$ T cells play a critical role in facilitating host adaptive immune responses. After maturation in the thymus, circulating $CD4^+$ T cells that have not encountered their specific antigen are termed naïve. Naïve $CD4^+$ T cells become activated by recognition of antigen presented by antigen presenting cells (APC) via the major histocompatibility complex (MHC) class II and differentiate into effector cells. Differentiation into specific subsets of T helper cells is based primarily on the antigen, the strength of the T cell receptor (TCR) signal, and the surrounding cytokines. Differentiation is orchestrated by expression of specific transcription factors

and subset specific secretion of a defined array of cytokines in response to the antigen (Reviewed in 1). Currently, there are many identified distinct lineages: T helper type 1 (Th1), T helper type 2 (Th2), interleukin (IL)-17 (IL-17) producing T helper (Th17) cells, IL-9 producing T helper (Th9) cells, IL-22 producing T helper (Th22) cells, T follicular helper (Tfh) cells, natural killer T (NKT) cells, and Treg cells. While the function of these T helper cells varies greatly, all express the CD4 co-receptor, and, with the exception of Treg cells, function to help activate other cells of the immune system.

III. Regulatory T cells

Treg cells play an essential role in immune homeostasis and maintenance of immunologic self-tolerance through suppression of pathologic and physiologic immune responses. The importance of Treg cell populations in immune homeostasis has been clearly identified through studies investigating Treg cell deficiencies which lead to a variety of autoimmune diseases, control of immune responses to infectious disease, and transplant rejection (2-4). However, Treg cell suppression can hinder immune responses during chronic infection and dampen anti-tumor responses. Thus, Treg cells play a dual role in immune regulation, and may be beneficial or detrimental in disease progression.

A. Foxp3 Expression

The majority of Treg cell populations can be identified by the expression of CD4 as well as the IL-2 receptor α-chain, CD25 (5, 6). Most Treg cells are $CD4^+CD25^+$, but subsets of Treg cells exist that have been identified as $CD4^+CD25^-$, or $CD8^+CD25^+$. However, the hallmark of most Treg cells is the expression of the functional-regulating transcription factor forkhead box p3 (Foxp3) (7-10). The importance of Foxp3 expression

in Treg cells has been demonstrated by the onset of autoimmune diseases, graft rejection, and reduced tumor growth in Foxp3-deficient mice (8, 11-13). While there is a strong correlation between $CD25^+$ and $Foxp3^+$ cells, subsets of T cells can be $CD4^+CD25^+Foxp3^-$, and likely represent activated effector cells (14). It is important to note that small subsets of suppressive T cells induced by cytokines may not gain Foxp3 expression, but instead exert suppression by secretion of IL-12, indicating the complexity in Treg cell subset classification and identification (Reviewed in 15).

B. Thymic Tregs

Despite thymic negative selection, the immune system maintains a population of self-reactive natural Treg (nTreg) cells. nTreg cells play an indispensable role in immune homeostasis, and the deficiency or dysfunction of naturally arising Treg cells is sufficient to cause autoimmune diseases in otherwise normal animals (5, 16, 17). nTreg cells generated in the thymus are antigen-primed and represent a functionally mature T cell population. Expression of the *foxp3* gene occurs early in thymic development before T cells are $CD4^+CD8^+$ double positive (18). Molecular mechanisms directing the transcription of the *foxp3* locus depend on DNA hypomethylation of conserved non-coding sequences (CNS), CNS1 and CNS2. Developing thymocyte expression of Foxp3 is dependent on both the strength and the duration of TCR stimulation (19-21). This stimulation results in epigenetic changes within the *foxp3* CNS2 region, in which several transcription factors, such as Ets1, Gata-3, signal transducer and activator of transcription-5 (STAT5), and Foxp3 itself assemble and activate Foxp3 expression (22-25). Demethylation of the *foxp3* CNS2 region results not only in increased gene

transcription, but also in nTreg stable expression of Foxp3 regardless of extracellular conditions (21, 26).

C. Induced Tregs

Treg cells can also be induced in peripheral tissues (iTreg), and share phenotypic expression of Foxp3, CD25, and cytotoxic T-lymphocyte antigen-4 (CTLA-4), but can differ in stability of expression of Foxp3. Induction of iTreg cells can occur *in vivo* and *in vitro* by a large number of factors such as continuous antigen stimulation, or stimulation by cytokines such as transforming-growth factor-β (TGF-β), macrophage-inhibiting factor-1, IL-2, IL-35, IL-10 or IL-4 (27-32). Upon stimulation by TGF-β and/or retinoic acid, histone modifications alter the CNS1 region of the *foxp3* locus, and enable several transcription factors such as nuclear factor of activated T cells (NFAT) and Smad, to assemble and transactivate Foxp3 expression (33, 34). However, this pathway does not alter methylation of the CNS2 region, thus resulting in distinct epigenetic patterns between nTreg and TGF-β-induced iTreg cells. In addition, TGF-β–induced iTreg cell expression of Foxp3 is less stable, evident by the loss of Foxp3 expression in the absence of TGF-β (35, 36). However, with the addition of IL-2, through the IL-2 receptor β-dependent STAT5 pathway, the CNS2 region of the *foxp3* locus is demethylated and results in iTreg stable expression of Foxp3 (37). Thus, while iTreg cell populations within the periphery can be induced from $CD4^+$ T cells by an array of extracellular cytokines, iTregs are primarily induced through TGF-β alone or together with IL-2.

D. Suppressive Mechanisms of Tregs

The suppressive mechanisms of Treg cells can vary depending on a variety of factors such as nTreg vs. iTreg cell subsets, expression of surface receptors and

transcription factors, the presence of extracellular cytokines, antigen stimulation, and proximity to target effector cells. Treg cell suppressive function can occur through direct cell-cell contact or indirectly by secretion of soluble factors such as indoleamine 2,3-dioxygenase (IDO), adenosine, and cytokines. Currently there are four described Treg cell 'modes of action', including suppression via: a) cytolysis; b) alteration of APC function; c) secretion of inhibitory cytokines and; d) metabolic disruption resulting in apoptosis.

While cytolysis of target cells is typically attributed to natural killer cells and cytotoxic $CD8^+$ T cells, Treg cells have also been shown to induce cytotoxicity in target cells by the production of granzyme A or B, in a perforin-dependent manner. Both nTreg cells and iTreg cell interactions with target cells such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes, and dendritic cells (DC) can induce cell death through the perforin/granzyme pathway independent of Fas/Fas ligand (38, 39).

Secondly, Treg cells can suppress APC function by physical blockade of T cell access to APCs by clustering in a lymphocyte function associated antigen-1 (LFA-1, $\alpha_L\beta_2$ integrin)-dependent manner (40). In addition, constitutive expression of CTLA-4 by Treg cells enables immunosupression by several mechanisms that require ligation of CTLA-4 by CD80 and CD86 on APCs: a) down regulation of CD80 and CD86 on mature DCs, b) inhibition of upregulation of CD80 and CD86 on immature DC, and/or c) production of IDO, which results in the production of immunosuppressive kynurenin (40, 41).

In addition to cell-cell contact, both nTreg and iTreg cells can exert suppressive function through the secretion of cytokines such as TGF-β and IL-10 (28, 42). As

previously discussed, iTreg cells can be induced from $CD4^+$ T cell in peripheral tissue in the presence of antigen stimulation and TGF-β. These iTreg cell populations in turn produce high amounts of TGF-β and moderate amounts of IL-4 and IL-10. Both IL-10 and TGF-β are potent anti-inflammatory mediators, and also function in the generation of other iTreg cells. Specific subsets of iTreg cells include IL-10-producing iTreg cells that are induced in the presence of antigen along with exogenous IL-10, and produce high levels of IL-10, moderate levels of TGF-β, IFN-γ, and IL-5. This specific $CD4^+CD25^-$ $Foxp3^-$, IL-10 high producing subset of iTreg cells have been termed T regulatory 1 (Tr1) cells (28). In addition, recent studies have demonstrated that iTreg cells can also be induced by IL-35 and IL-10 resulting in a subpopulation of IL-35 producing Treg (iTr35) cells that are functionally suppressive but $Foxp3^-$ in both humans and mice (29, 43-45).

Treg cells suppressive mechanisms also include metabolic disruption of effector target cells through several pathways. Although highly debated, some studies suggest that high expression of the IL-2 receptor (IL-2R) by Treg cells enables the binding of soluble IL-2, thus depriving actively dividing effector cells of IL-2 signaling necessary for proliferation (46, 47). Other studies suggest that IL-2 deprivation alone is not required for Treg cell suppressive function (48-50). Rather than IL-2 depletion, several studies have indicated that contact-dependent IL-2 suppression in target cells can result in suppression. Specifically, upon activation of Treg cell TCRs, they can suppress $CD4^+$ T cells and $CD8^+$ T cell proliferation by inhibiting IL-2 mRNA transcription in a cell-contact-dependent, cytokine-independent mechanism (46, 51-53). Treg cells can also exert suppressive function by pericellular adenosine production, which binds to and activates the adenosine A2A receptor of effector T cells and suppresses effector T cell function

(54). In addition, metabolic disruption by Treg cells can also include direct transfer of cyclic AMP, a potent inhibitory second messenger, directly into effector T cells via gap junctions (55). Taken together, Treg cells can mediate suppression through several mechanisms that include cell-cell contact or secretion of soluble factors, both which require Treg cells to be in close proximity to the effector cells they suppress.

IV. Lymphocyte-Endothelial Interactions

The dynamic nature of the immune system provides the unique capability for surveillance as well as local and specific responses to tissue damage, pathogens, and transformed cells. Leukocyte migration into peripheral tissues was discovered as early as the 1880's by Pfeffer and Leber who found that chemotatic substances were capable of attracting leukocytes into tissues (56, 57). Almost 60 years ago, Gowans first described the ability of immune cells to recirculate from the blood to lymphoid tissue (58, 59). Later, Gowans and Knight further described lymphocyte migration through specialized post-capillary high endothelial venules (HEV) which are present in all secondary lymphoid tissue with the exception of the spleen (60). These landmark discoveries have pioneered research in leukocyte movement between blood, lymphoid tissue, and peripheral tissue over the last 130 years.

Leukocyte migration from the blood to peripheral lymphoid and non-lymphoid tissue is mediated by interactions between the leukocytes and endothelial cells that line blood vessel walls. Interactions between leukocytes vary greatly depending on specific expression of molecules both on the leukocyte and the endothelium. Migration of leukocytes can be highly specific, directing only subsets of cells into particular locations,

and at specific times. This specificity in migratory ability and patterns relies on the regulation of expression and/or cleavage of surface molecules present on leukocytes and their cognate ligands on the vascular endothelium. In addition, chemotactic gradients, hemodynamic forces, vascular permeability, and cell signaling all play a role in the complex process of leukocyte migration.

A. Leukocyte Adhesion Cascade

Adhesion molecules are proteins present on the surface of leukocytes that interact with specific ligands on the endothelium in a process known as the adhesion cascade (Fig. 1). Initial capture of leukocytes out of the rapidly moving blood stream is mediated by the selectin family of adhesion molecules that bind to the mucin family of ligands causing the cells to tether (capture) and roll along the endothelium (61, 62). During rolling, chemokines secreted by cells such as resident macrophages and endothelial cells, bind to chemokine receptors on the rolling leukocyte initiating a series of G-protein signal transduction pathways such as the Ras/Raf/MAPK pathway (63). Chemokines play a significant role in subsequent activation of other adhesion molecules such as integrins by inducing a conformational change to a high affinity binding state, thereby enabling them to strongly interact with their ligands, the immunoglobulin (Ig) superfamily molecules (64-69). Select integrin-Ig binding can facilitate capture and support the rolling of leukocytes at physiologic shear in the absence of selectins but is dependent on the density of Ig family members (70, 71). However, optimal function of integrins occurs after leukocytes have been first captured and their velocity has been reduced by the selectin family thus allowing the integrin-Ig interaction to facilitate even slower rolling

velocities and eventually firm arrest (72, 73). Upon arrest, the cell then migrates either between or through endothelial cells to target sites within the tissue (74-77).

B. L-selectin

The selectin family members, P- and L-selectin have been shown to function early during the leukocyte adhesion cascade to capture leukocytes from the rapidly moving blood. L-selectin is constitutively expressed on all classes of leukocytes and plays an important role in mediating capture of leukocytes from the blood stream and also supports rolling on the vascular endothelium independent of E- or P-selectins (78-81). The importance of L-selectin in naïve and memory lymphocyte migration is demonstrated by an ~90% reduction in lymphocyte migration to lymph nodes in L-selectin-deficient (L-selectin$^{-/-}$) mice (82). L-selectin functions in the initial capture of leukocytes, but also participates throughout other steps of the adhesion cascade (83). Importantly, L-selectin contains an endoproteolytic cleavage site that is responsible for the rapid cleavage from the surface of the lymphocyte upon activation (84). This cleavage of L-selectin is important in downregulating inflammatory responses and results in the presence of high levels of soluable L-selectin within the blood (85).

C. L-selectin Ligands

L-selectin-dependent migration is appropriately maintained through the regulated expression of L-selectin ligands on the endothelium as well as cleavage of L-selectin from the leukocyte surface. L-selectin ligands encompass a vast array of mucin family molecules, many of which have been identified (e.g., CD34, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), peripheral node addressin (PNAd), P-selectin glycoprotein ligand-1 (PSGL-1), and human endothelial cell antigen-452 (HECA-452)),

but it is likely that other ligands have yet to be identified. These known L-selectin ligands consist of protein scaffolds that are broadly expressed, but only through appropriate post-translational modifications of a protein core do they become functional L-selectin ligands. These modifications such as glycosylation, fucosylation, and sialylation produce a tetrasaccharide 6-sulfo sialyl Lewis X capping group that serves as the primary ligand for L-selectin and can be identified by a monoclonal antibody (mAb) MECA-79 (86-88). In addition to these modifications, high avidity binding to L-selectin has been shown to require sulfation on oligosaccharide branches by sulfotransferases GlcNAc6ST-1 and -2 (89-92). In addition to sulfotransferases, other transferases that have been shown to generate functional L-selectin ligands include core 2 β1, 6-N-acetylglucosaminlytransferase, as well as β1,4-galactosyltransferase-I and -IV, fucosyltransferase-VII and -IV, and α2,3-sailyltransferase IV (92-97).

D. α4 integrins

The integrin family of adhesion molecules consists of a heterodimeric complex made up of an α and β chain. Integrins are expressed on the surface of numerous classes of cells and are important in many cellular functions such as movement, signaling, and anchoring of the cell to the extracellular matrix (98-100). Integrins play an important role in migration of leukocytes through HEV, inflamed endothelium, and mucosal lymphoid tissue. There are many combinations of integrin subunits, giving rise to a vast array of integrins with a diversity of functions. Two classes of β integrins, β_7 and β_1 pair with the α_4 integrin subunit to give rise to $\alpha_4\beta_7$ integrin and $\alpha_4\beta_1$ (very late antigen-4, VLA-4) integrin. $\alpha_4\beta_7$ integrin is constitutively expressed on naïve T and B cells independent of

localization within lymphoid tissue and functions to regulate lymphocyte migration through binding specificity to vascular cell adhesion molecules.

$\alpha_4\beta_7$ integrin is expressed by most lymphocytes and supports migration to gut mucosal tissue such as the lamina propria, and mucosal-associated lymphoid tissues including the Peyer's patches and mesenteric lymph nodes (MLN) through binding to its primary ligand MAdCAM-1 (101-105). Importantly, $\alpha_4\beta_7$ integrin can also mediate binding to a secondary ligand, vascular cell adhesion molecule-1 (VCAM-1) expressed on inflamed endothelium; however, this interaction has not been well defined (106-109). Interestingly, it has recently been shown that $\alpha_4\beta_7$ integrin-mediated binding to VCAM-1 was essential for the migration of T cells to sites of cutaneous inflammation during skin contact hypersensitivity responses (110). However, the extent of $\alpha_4\beta_7$ integrin-mediated binding to VCAM-1 in lymphocyte subset migration into sites of inflammation has yet to be elucidated.

VLA-4 is involved in binding to VCAM-1 and supports leukocyte migration to sites of inflammation (106, 111-113). VLA-4 is highly expressed on monocytes and is important for their migration to sites of inflammation after the initial surge of neutrophils (114, 115). VLA-4 is also expressed on the surface of subsets of effector and memory T cells and functions to mediate their migration to sites of inflammation (116, 117). Interestingly, while α_4 integrin chains can pair with β_1 or β_7 chains, one study suggested that the β_1 integrin chain preferentially associates with and outcompetes β_7 pairing with the α_4 integrin chain, suggesting that if both β_1 and β_7 integrin chains are present, VLA-4 will be the dominant adhesion molecule expressed (118). Taken together, lymphocyte subset expression of $\alpha_4\beta_7$ and/or $\alpha_4\beta_1$ integrin is important for binding to distinct

vascular-expressed cell adhesion molecules and can direct lymphocyte homing to mucosal or inflamed tissues.

F. Cooperative Adhesion molecule interactions

It is recognized that adhesion molecules function in a complex series of overlapping interactions that are mediated by chemokines and vascular adhesion molecule expression. For example, optimal selectin-mediated rolling is facilitated by integrin LFA-1 binding to intercellular cell adhesion molecule-1 (ICAM-1) during inflammation by stabilizing leukocyte/endothelial cell interactions (119-123). Specifically, L-selectin-mediated rolling is optimized in the presence of ICAM-1, while inversely, LFA-1/ICAM-1 interactions are primed by L-selectin signaling via ligation of the lectin domain (119, 120, 124, 125). Furthermore, cooperation between L-selectin and LFA-1/ICAM-1 function *in vitro* and *in vivo*, results in slower lymphocyte rolling velocities than L-selectin-mediated rolling velocities alone (119, 126). Synergistic interactions have also been demonstrated between L-selectin function and $\alpha_4\beta_7$ integrins in optimal lymphocyte migration to gut associated lymphoid tissue where MAdCAM-1 is expressed (127, 128). Collectively, these studies indicate the complexity of overlapping function between adhesion molecules during lymphocyte migration and recirculation. It is unclear, however, if L-selectin also functions with $\alpha_4\beta_7$ integrin to enhance recruitment of leukocytes to sites of inflammation.

V. Lymphocyte Migration and Recirculation

After maturation in the thymus, mature naïve T cells exit the thymus and enter the blood stream. Naïve T cells home to lymph nodes, spleen, or mucosal tissue by entry

through HEVs. Within secondary lymphoid tissue, naïve T cells migrate to T cell zones where they may recognize antigens presented by professional APCs. Naïve T cells that do not recognize antigen remain naïve and exit lymphoid tissue and reenter the blood stream by afferent lymphatics or directly enter the circulation from the spleen. This pattern of trafficking from the blood to lymphoid tissue enables continual recirculation of lymphocytes throughout the body.

Upon recognition of antigen, naïve T cells become activated, differentiate into effector T cells, and undergo rapid proliferation. Effector T cells may respond to target antigen within the lymph node, or may exit the lymph nodes or spleen and reenter the circulation. Circulating effector T cells preferentially home to peripheral tissue and sites of inflammation where they can ellicit immune responses. Regulation of lymphocyte recirculation and migration to secondary lymphoid tissue or peripheral tissue is mediated by adhesion molecule expression, vascular expression of ligands, shear stress of the moving blood, and chemokine activation. Through these mechanisms, lymphocyte migration and homing can be tightly regulated and subset specific.

A. Migration to secondary lymphoid tissue

Lymphocyte migration into lymph nodes primarily occurs from the blood through HEV (Fig. 2A). Migration into lymph nodes and Peyer's patches is dependent on lymphocyte expression of L-selectin and its ligands expressed on HEVs (129). HEV-expressed L-selectin ligands are collectively termed PNAds. Many PNAds can be identified by the monoclonal antibody MECA-79 which recognizes the carbohydrate decorations on protein backbones, 6-sulfo sialyl Lewis^x and non-sialyl Lewis^x of Core 1 branches (130). In mucosal-associated lymphoid tissue such as MLN and Peyer's patches,

lymphocyte migration through HEV is also mediated by expression of MAdCAM-1, which is not expressed on HEV in peripheral lymph nodes (PLNs, 131). Interactions between $\alpha_4\beta_7$ integrin and MAdCAM-1 enables capture, rolling, and adhesion of lymphocytes on HEV in mucosal lymphoid tissue. This interaction is one example of subset specific homing of $\alpha_4\beta_7$-expressing lymphocytes (such as gut-associated memory cells) to mucosal tissue. If properly glycosylated, MAdCAM-1 can also serve as a ligand for L-selectin, enabling L-selectin-dependent adhesion in the MLN (132). Thus, MAdCAM-1 expression directs both naïve T cell as well as specific $\alpha_4\beta_7$-expressing lymphocyte subset homing into mucosal lymphoid tissue.

Lymphocyte homing to secondary lymphoid tissues is also directed by chemokine receptor expression on lymphocytes that bind to chemokines displayed on endothelial cells of postcapillary venules. Specifically, lymphocyte homing to lymph nodes and Peyer's patches is mediated by CC-chemokine receptor 7 (CCR7), expressed on lymphocytes, binding to HEV chemokines such as secondary lymphoid-tissue chemokine (SLC) and EB11 ligand chemokine (ELC) (133-136). CCR7 binding to SLC mediates intracellular signaling that induces LFA-1 high affinity binding states as well as increases in L-selectin ligand binding activity (137-140).

Chemokine-chemokine receptor binding initiates intracellular signaling that results in integrin conformational changes to high affinity binding states (64, 65, 141). LFA-1, expressed by all classes of leukocytes, directs migration of lymphocytes into lymph nodes and Peyer's patches through binding to its ligand ICAM-1 (142, 143). ICAM-1 is expressed at moderate level on HEVs and is required for optimal migration of lymphocytes into lymph nodes and Peyer's patches (144, 145). Taken together,

lymphocyte migration into lymph nodes and Peyer's patches is coordinated by lymphocyte adhesion molecule and chemokine receptor expression, and by the appropriate ligands and chemokines expressed on the HEVs.

B. Migration to peripheral tissues

Subset-specific lymphocyte homing provides a mechanism for separating specialized immune responses distinctive of cutaneous, systemic, or intestinal responses. Through differential expression of adhesion molecules and chemokine receptors, specialized lymphocyte subsets can be targeted to migrate to specific immune microenvironments. Lymphocyte subset-specific migration to inflamed peripheral tissue is directed not only by effector T cell adhesion molecule and chemokine receptor expression, but also adhesion molecule ligands and chemokines present on the inflamed endothelium (Fig. 2B). Tissue-specific tropism was first identified in early studies that described adoptively transferred lymphocytes preferentially migrated to the tissues from which they were isolated (146-148). Later studies further defined preferential homing of two T cell subsets based upon expression of adhesion molecules such as cutaneous lymphocyte antigen-1 (CLA-1) or $\alpha_4\beta_7$ integrin which directed their migration to cutaneous or intestinal tissue, respectively (Reviewed in 149). Importantly, lymphocyte migration to sites of inflammation is largely regulated by adhesion molecules expressed by inflamed vascular endothelium. In addition, chemokine receptor expression also serves as an important mediator of tissue-specific migration (Reviewed in 150).

C. Selectins (and ligands) in inflammation

Lymphocyte homing to tissues is dependent on selectin-mediated capture and rolling of lymphocytes along vascular endothelium. P- and E-selectin are not normally

expressed by resting vascular endothelium, but are upregulated in response to inflammatory stimuli. P-selectin is stored in cytoplasmic granules and can be rapidly transported to the surface of endothelial cells in response to inflammatory stimuli such as histamine, thrombin, complement factors, cytokines, and free radicals (61). E-selectin upregulation on cytokine-activated endothelial cells requires new protein synthesis and may function as a homing receptor for specific T cell subsets (61, 151, 152). Lymphocyte expression and proper glycosylation of PSGL-1 facilitates binding of lymphocytes to L-, P- and E-selectins (153). PSGL-1 glycosyltransferases are induced in T cells and require antigen-stimulated activation of T cells (154-159). Importantly, L-selectin expression can also mediate tissue-specific homing by binding to vascular endothelium-expressed ligands such as glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, Sgp200, podocalyxin, and endomucin. In addition, L-selectin can mediate secondary tether formation by binding to ligands such as PSGL-1, endoglycan and hematopoetic cell E- and L-selectin ligand (130, 160-163).

D. Vascular expression of cell adhesion molecules during inflammation

Migration of lymphocytes to inflamed tissues is initiated by innate immune responses that result in cytokine-induced expression of E-selectin, P-selectin, and integrin ligands by endothelial cells. In addition to E- and P-selectin upregulation on inflamed endothelium, members of the Ig superfamily are critical for integrin binding and leukocyte firm arrest at sites of inflammation. The Ig superfamily consists of adhesion molecules with variable numbers of Ig domains that serve as ligands for the integrin family. These include the cell adhesion molecule group of adhesion molecules expressed on the endothelium. During inflammation, the primary Ig superfamily molecules that

regulate lymphocyte migration are ICAM-1, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and VCAM-1. ICAM-1 and VCAM-1 mediate adhesion through binding of integrins present on leukocytes, while PECAM-1 can mediate adhesion through homophilic or heterophilic interactions and modulates migration of leukocytes through vascular endothelium via intercellular junctions (164, 165). In addition to ICAM-1 expression on HEV, basal low levels of ICAM-1 on resting vascular endothelium are upregulated in response to inflammatory stimuli such as inflammatory cytokines, lipopolysaccharides, or phorbol esters (166-168).

VCAM-1 was first identified as a LFA-1-independent ligand expressed by endothelial cells in the recruitment of monocytes to inflammation (169). Endothelial cells transcriptionally upregulate VCAM-1 in response to inflammatory cytokines and thrombin (170, 171). VCAM-1 functions in the recruitment of leukocytes to tissue during the late stages of inflammation through binding to its primary integrin receptor VLA-4. This recruitment is mediated by the activation of VLA-4 on the surface of monocytes and subsets of lymphocytes that bind to VCAM-1 on inflamed endothelium (106, 107, 172-174). However, VCAM-1 can also serve as a ligand for $\alpha_4\beta_7$ integrin in lymphocyte recruitment to cutaneous inflammation. In fact, a recent study by Ohmatsu et al. described lymphocyte Th1 effector cell migration during cutaneous inflammation was inhibited in β_7 integrin-deficient mice, indicating $\alpha_4\beta_7$ integrin-dependent migration during inflammation (110). Thus, vascular expressed cell adhesion molecules are key mediators of lymphocyte trafficking to sites of inflammation.

E. Cutaneous tissue homing directed by chemokines

Similar to chemokine-directed migration into lymph nodes, chemokines also facilitate lymphocyte homing to sites of cutaneous inflammation. CCR4 is expressed by most circulating CLA-1$^+$CD4$^+$ T cells, and its ligand, CCL17, is expressed by keritinocytes and venules within the skin, and is upregulated during inflammation (175, 176). Another skin-homing chemokine receptor, CCR10, is expressed by a subset of CLA-1$^+$CD4$^+$ T cells and binds to its ligand CCL27 expressed by skin keratinocytes (176, 177). However, CCR4 alone is not sufficient for T cell homing to the skin, evident in studies identifying CCR4 expression by non-skin homing lymphocytes such as $\alpha_4\beta_7$ integrin$^+$ effector T cells, as well as T cells resident in the lungs of patients with asthma, and synovial fluid of rheumatoid arthritis patients (178-181). While these chemokine receptors have been shown to play an important role in T cell-specific homing to tissues, there is a high degree of chemokine receptor co-expression and redundancy, indicating complex and specific regulation of T cell homing to extralymphoid tissue.

F. Dendritic cell priming for tissue specific homing

Differential adhesion molecule and chemokine receptor expression of effector T cell subsets depends on the environment in which the T cell is activated (Reviewed in 150). It was first thought the environment of the lymph node in which T cells were activated influenced their homing specificities. However, within the same lymph node, multiple effector T cell subsets with distinct homing specificities can be generated (158). It has been found that DCs within the lymph node are responsible for the induction of chemokine receptor and adhesion molecule expression of the T cells which they activate. In fact, DC secretion of retinoic acid induces T cells to upregulate $\alpha_4\beta_7$ integrin and CCR9, whereas IL-12 secretion by DCs induces fucosyltransferase-VII (FtVII)

expression and CCR4, and UVB-induced vitamin D3 ($1,25(OH)_2D3$) can induce expression of IL-10 (182-188). Thus, expression of adhesion molecules on lymphocytes can depend on DC 'imprinting', based upon the specific environment from which the DCs were initially activated.

G. Treg cell migration

Migration of Treg cells to sites of inflammation is critically important to their suppressive function since secretion of cytokines and cell-cell contact of Treg cells with effector T cells and APCs is necessary for Treg cells to exert suppressive effects (189). It was shown that both $CD4^+$ and Treg cells are primed in the same draining lymph nodes during tumor progression (189). In addition, paracrine signaling of the immunosuppressive cytokines IL-10 and TGF-β requires close proximity of Treg cells to their suppressive targets (190). Treg cell migration into secondary lymphoid tissue is controlled by chemokines, chemokine receptors, and adhesion molecules (191, 192). Chemokine receptors (i.e. CCR4, CCR5, and CCR8) expressed by Treg cells bind to chemokines (i.e. CCL17, CCL4, CCL1) produced by activated B cells, DCs, monocytes, and activated T cells (193-195). Treg cells are also present in non-lymphoid sites, including autoimmune lesions, cutaneous sites of inflammation, and tumors (196).

Adhesion molecule expression for subsets of Treg cells has been characterized and shows that the predominant population of $CD4^+CD25^+Foxp3^+$ Treg cells express high levels of L-selectin (192, 197). Interestingly, although Treg cell and conventional T cells both require L-selectin for migration, and surface expression and density of L-selectin are similar, it was found that Treg cell L-selectin turnover rates were significantly higher and they showed an ~3-9 fold lower migration rate into peripheral

lymph nodes than their conventional $CD4^+$ T cell counterparts (192). As previously discussed, L-selectin is important for migration to lymphoid tissue such as draining lymph nodes as well as to sites of inflammation. In addition to L-selectin, Treg cells display other adhesion molecules in diverse phenotypic patterns that divide them into complex subsets based on differential expression of these molecules. Specifically, Treg cells are reported to have high VLA-4 and $\alpha_4\beta_7$ integrin expression (198). However, a study on human Treg cells showed that this expression pattern was not from a single population but rather from two distinct populations of Treg cells expressing high levels of either VLA-4 or $\alpha_4\beta_7$ integrin (199). Thus, while some information on the expression patterns of adhesion molecules for Treg cells exists, little is known about the individual or collective contributions of these adhesion molecules to the migration of Treg cells. The migration of Treg cells to tumor sites, surrounding tissue, and draining lymph nodes plays an important role in the establishment and progression of tumors. Therefore, understanding the role of adhesion molecules, such as L-selectin, in regulating Treg migratory patterns *in vivo* may provide new insights into tumor therapy.

VI. Hemodynamics

The initiation of the adhesion cascade, and the subsequent events that lead to leukocyte transendothelial migration require leukocytes to overcome the shear forces exerted on the cells within the blood. Within the microcirculation, the flow of blood is controlled through arteriolar, capillary, and venule dilation, and the distribution and volume of blood is regulated based upon the metabolic needs of the tissue. Pressure-flow relationships within microcirculation have been an intensive field of research since the

early 1840's when Poiseuille first observed the separation of plasma and cells in arterioles and venules (200, 201). While much work on the pressure and flow mechanics that govern blood flow in microcirculation center around skeletal and smooth muscle blood supply and metabolic demands, blood flow rates are also essential regulators of leukocyte recirculation and migration. Leukocyte migration out of the blood stream requires the ability of adhesion molecules to a) rapidly interact with endothelial ligands; b) bind to ligands under conditions of shear forces; and c) reduce velocity and enable firm adhesion of cells in the presence of blood shear forces. Hence, hemodynamic factors that govern blood flow through HEV, tissue post-capillary venules, and inflammatory sites play a critical role in leukocyte recirculation and migration.

A. Rheological properties of blood

The rheological behavior of blood has proved to be a complicated field of intensive and ongoing research (Reviewed in 202). Early intravital studies by Landis attempted to use Poiseuille's law to explain microvascular resistance (203). Fåhraeus discovered blood viscosity was reduced due to reductions in hematocrit as blood moves into smaller diameter vessels and his work led to the current understanding of the properties of 'apparent viscocity' of blood, meaning that blood viscosity varies based upon shear rate and temperature (204). This supported further by supplemented by Vejlen's research on blood flow affected by leukocyte behavior within the microcirculation and Krogh's work on flow distribution through capillaries and sequestration of red blood cells under normal and pathological conditions (205, 206). These pioneering studies lead the way to our current understanding of the fundamental properties of blood and microcirculation. Resistance to flow is governed by factors such

as cellular concentration of the blood, red blood cell and leukocyte deformability, and leukocyte adhesion to vasculature, however the extent to which these factors regulate resistance is determined by vessel branching and diameter (or extent of dilation) of the blood vessel itself (Reviewed in 202).

B. Blood Flow in Microvasculature

Blood flow within the microvasculature can be explained, in part, by Poiseuille's law explaining that larger particles (or cells) are forced to central parts of a flowing stream, and this is amplified at higher flow rates (207). This has been demonstrated for leukocytes *in vitro* and *in vivo*, where larger leukocytes are forced to the center of the stream at higher shear rates (208, 209). However, when blood shear rates are slowed, leukocytes are able to move to the marginal region of flow near the vessel wall and thus increases the probability of leukocyte interaction with venule endothelium (210). After blood exits the capillary into post-capillary venules, the diameter of the vessel increases and thus flow rates are reduced. Most leukocyte extravasation from the blood stream occurs in post-capillary venules, where the shear rates near the blood vessel wall can range from 35 s^{-1} to 560 s^{-1} or a wall shear stress of 0.25-4 dyne/cm^2 (211). In steady state post-capillary venules, physiologic shear stress ranges from 1.0-2.0 dyne/cm^2. However, vessel wall shear stress depends on many factors including vessel diameter, flow rate, and vessel occlusions (Reviewed in 202). For example, low shear stress (0.25-0.75 dyne/cm^2) can occur in occluded vessels where shear flow can be disrupted by blockage of the lumen or during inflammation, in which blood vessels dilate to allow for increased blood flow (212-214).

C. Hemodynamic regulation of Adhesion Molecule Interactions

As previously discussed, initial capture of leukocytes is primarily mediated by the selectin family of adhesion molecules that bind to mucin family ligands causing the cells to tether and roll along the endothelium (61, 62). Selectin binding occurs in a shear-dependent manner in which optimal interactions occur in a range of 1.5-2.8 dyne/cm^2 which is characteristic of post-capillary venules (212). Selectin-ligand binding involves catch-bond kinetics in which the rapid formation and breakage of bonds is optimized at high shear forces with association/dissociation rates ($K_{on/off}$) (215-218). Upon leukocyte capture from the blood stream, selectin-ligand interactions are resistant to shear forces based upon high tensile strength, evident by the ability of leukocytes with low selectin density to tether and resist premature dissociation by increasing shear forces (219). Shear-resistant rolling enables leukocytes to interact with the endothelium for longer intervals, thereby enhances conversion to integrin-mediated adhesion and eventually transendothelial migration (220).

Shear stress-dependent leukocyte interactions with endothelium are not limited, however, to the selectin family of adhesion molecules. In the absence of selectins, optimal integrin interactions can occur only at low physiologic shear stress (71, 221, 222). By contrast, a few studies have reported selectin-independent, integrin-mediated capture and adhesion at physiologic shear stress (70). However, the number of cells that interacted in these studies was severely reduced in the absence of selectins. In addition, shear stress alone can activate integrins, and has been shown to be required for chemokine-induced activation of integrins on lymphocytes (223, 224).

VIII. Tumor Immunology

The tumor microenvironment maintains a multitude of mechanisms that mediate immune suppression and angiogenesis. Tumor survival is dependent on evasion of the immune system, while growth and progression are dependent on nutrient and oxygen supply. Many of the mechanisms by which tumors suppress immune responses are part of the physiologic mechanisms that also regulate homeostasis. Formation and repair of blood and lymphatic vessels occurs after injury and ensure proper delivery of oxygen and nutrients to tissues and drainage of lymph. The ability of tumors to hijack these mechanisms results in tumor growth, inefficient and suppressed immune responses to tumors, metastasis, and inhibition of immunotherapy.

Tumor growth and progression are dependent on the ability of tumor cells to rapidly proliferate as well as to evade immune responses. Tumors have many mechanisms to evade immune responses and poor immunogenicity of tumors enables tumors to go undetected or to proliferate faster than effector cells can respond. In addition, immune evasion by tumors can occur through mechanisms such as masking, internalizing, or modifying surface antigens (Reviewed in 225). Importantly, tumors can directly affect anti-tumor immune responses by secretion of anti-inflammatory and immunosuppressive cytokines present within the tumor itself and within the local tumor-draining lymph node, as well as by generating tumor-protective immune suppressor populations such as myeloid derived suppressor cells (MDSCs) and Treg cells.

A. Tumor antigenicity

Negative selection during lymphocyte maturation in the thymus helps ensure that lymphocytes do not recognize self antigens. This presents a difficult obstacle in tumor

cell recognition by lymphocytes due to the tumor cell origin as "self". However, transformed cells often express antigens that are not found on normal cells, and thus allow immune recognition. The antigenicity of tumors varies greatly depending on the type, stage, and location of the tumor. Tumor-associated antigens can also be downregulated in many cancers, along with secretion of inhibitory molecules that dampen immune responses. Tumor-associated antigens that can be recognized by the immune system result from over or aberrent expression of naturally occurring cellular proteins, mutated tumor-specific proteins, proteins derived from onogenic viruses (eg. human papillomavirus), or products of onogenes or mutated oncosuppressors (226, 227). A few strong immunogenic tumors such as melanomas and renal cell carcinomas induce robust immune responses, while many tumors are poorly immunogenic such as leukemias, lymphomas, colorectal cancers, and carcinomas of hepatocellular, pancreatic, and prostate origin (228, 229).

B. Immune activation

Adaptive immune responses to tumors include a wide range of cellular responses. Tumor-associated antigens are presented by MHC class I on tumor cells and directly activate $CD8^+$ T cells. Upon activation, $CD8^+$ T cells, along with natural killer (NK) cells, mediate apoptosis in target cells by expression of Fas ligand leading to activation of the Fas/FasL apoptotic pathway. Tumor antigens can also be presented on the surface of APCs by MHC class II molecules and result in the activation of $CD4^+$ T cells. Upon activation, $CD4^+$ T cells proliferate, secrete cytokines, and activate other cells of the immune response such as $CD8^+$ T cells, macrophages, B cells, and other $CD4^+$ T cells.

C. Tumor-infiltrating lymphocytes

Immune responses to tumors can occur within secondary lymphoid tissue or within the tumor by tumor-infiltrating lymphocytes (TILs) (230-233). TILs have been an attractive target for immunotherapy since early studies in animals showed adoptively transferred cells from immunized donors could mediate tumor regression and that IL-2 could be used to expand these cells (234-236). In 1986, Rosenberg and colleagues demonstrated regression of metastases in mice resulted from the combination of TILs and cyclophosphamide, and further demonstrated that TILs could induce cancer regression in human patients with metastatic melanoma (237, 238). Within the tumor, TILs represent a heterogeneous population of lympohcytes that consist of antigen-primed effector $CD8^+$ and $CD4^+$ T cells (239). However, infiltration by TILs, even along with other anti-tumor cells such as macrophages and natural killer cells, is often ineffective at mediating tumor eradication.

C. Immune Suppression by MDSCs

The chronic inflammatory microenvironments of tumors activate myelopoiesis resulting in the expansion and recruitment of immature myeloid cells (240-242). These MDSC populations without lymphocyte lineage markers were initially described as natural suppressors cells that could functionally suppress lymphocyte responses to antigens (243-245). MDSCs are immature myeloid cells that have a broad range of phenotypes in both humans and mice. Increased production of intratumoral granulocyte (G-CSF) or granulocyte-macrophage (GM-CSF) colony stimulating factors drive accumulation of granulocytic MDSCs (G-MDSC) or monocytic MDSCs (M-MDSCs), respectively (242, 246, 247). The abundance and suppressive mechanisms of G- and M-MDSCs differ, with a higher abundancy of G-MDSC in both human and murine cancers.

G-MDSCs modulate immune suppression predominantly by production of reactive oxygen species (248). On the other hand, M-MDSCs are less abundant, but potent, immunosuppressors through the production of nitric oxide and arginase-1 (246, 249, 250).

D. Role of Tregs in Cancer

Treg cells have an immense impact on tumor development, growth, and immune response to tumor-associated antigens. Studies have clearly demonstrated elevated proportions of $CD4^+$ T cells in the population of TILs of tumor masses and tumor-draining lymph nodes (189, 251-255). In addition, chronic stimulation favors the generation of nTreg cells *in vivo* (256, 257). It has also recently been shown that the overall number of Treg cells is permanently upregulated in cancer patients even after cancer eradication (258). Accumulation of Treg cells at tumor sites and tumor-draining lymph nodes impedes the generation and activation of tumor-specific effector T cell responses to tumor tissues (259-266) and cancer immunotherapy (267). The origin of Treg cells at tumor sites remains ambiguous, however many studies have suggested that Treg populations arise from conventional T cells in the cytokine milieu of tumor environments (14, 268, 269) or after interaction with nTreg cells (270).

Both nTreg cells and iTreg cells contribute to tumor-specific T cell tolerance. However, pre-existing nTreg cells are neither required for the induction of iTreg cells nor measurably impact the extent of their accumulation. Instead, induction of Ag-specific regulatory cells from naive T cells is intrinsically influenced by the tumor microenvironment (271). The homing and migration of nTreg cells into tumor tissues may contribute to Treg cell accumulation, however the induction of iTreg cells within the

tumor or draining lymph nodes may largely facilitate the overall accumulation of pro-tumorigenic Treg cells (272, 273).

E. Cytokines within the tumor microenvironment

Tumor microenvironments are established when cancer cells release cytokines and growth factors into the surrounding area and reprogram many other types of cells thereby creating an immunosuppressive environment (274, 275). Tumors host a large number of cells, including endothelial cells, fibroblasts, TILs, and other inflammatory cells that infiltrate the tumors and also produce cytokines that modulate pro- or anti-tumorigenic responses (276-280). Within the tumor microenvironment, the milieu of cytokines and cytokine-producing cells creates a complex environment regulating the development of T cells (281-283). Changes in cytokine milieus may ultimately be responsible for the overall progression or rejection of the tumor (284). For example, some studies have illustrated synergistic and antagonistic roles of cytokines such as IL-2, IL-9, IL-10, IL-21, IL-23, and IL-35 on the development of Treg or inflammatory Th17 cells (285-289). Two immune suppressive cytokines that play a role in Treg cell development and immune suppression, TGF-β, and IL-10 are discussed briefly below.

Specifically, the immunosuppressive cytokines TGF-β and IL-10 are important cytokines in the regulation of immune responses within tumors. TGF-β is a pleiotropic cytokine which can act as a potent immunosuppressor of interferon-γ (IFN-γ) production by $CD4^+$ and $CD8^+$ T cells as well as induce the generation of Treg cell populations (290, 291). TFG-β produced within the tumor microenvironment by tumor-associated macrophages and Treg cells can also inhibit macrophage activation and production of IL-6, TNF-α, and IL-1β (42, 292, 293). IL-10 is another important immunosuppressive

cytokine produced by tumor cells, macrophages, and Treg cells that suppresses immune responses and promotes tumor immune evasion (294). In addition to immune suppression, the presence of IL-10 has been shown to stimulate development of Burkitt's lymphoma, and has been correlated with poor prognosis in diffuse large B-cell lymphoma patients (295, 296). Thus, TGF-β and IL-10 facilitate immunosupression as well as facilitate the generation and function of Treg cells. However, due to the complexity of tumor microenvironments, defining the role of individual cytokines on the development of pro- or anti-tumor immune responses remains a challenging task.

F. Angiogenesis

The formation of new blood vessels to deliver oxygen and nutrients is essential to tumor growth. Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is primarily mediated by secretion of the pro-angiogenic factor vascular endothelial growth factor (VEGF). As tumors develop, hypoxic conditions in the center of the tumor can induce expression of transcription factors such as hypoxia-inducible factor-α (297). In addition, activation of oncogenic proteins such as epidermal growth factor receptor, and proteins of the RAS/RAF/MEK/ERK pathway can also control expression of VEGF (298, 299). Pathological angiogenesis stimulated by hypoxia or pro-angiogenic oncoproteins results in the proliferation and migration of vascular cells and the formation of new blood vessels. However, due to poor structural development of tumor microvessels resulting in thin walls, underdeveloped endothelial cell-cell junctions, abnormal basement membranes, and discontinuous smooth muscle cells and pericytes, these new blood vessels are often hyperpermeable (300-302). Hyperpermeability of blood vessels has been correlated with tumor transendothelial migration, and increased tumor

malignancies (303). In addition, during pathological angiogenesis, the newly formed blood vessels are enlarged, have irregular blood flow, and can result in microhemorrages within the tumor (Reviewed in 304).

In addition to angiogenesis, the production of VEGF can modulate downregulation of adhesion molecules such as ICAM-1 on endothelial cells, resulting in decreased leukocyte infiltration (305). Furthermore, VEGF-dependent mechanisms can also stimulate the generation of MDSCs and Treg cells, prevent DC maturation, and reduce splenic T cell numbers, frequency, and function (306-309). Thus, VEGF is an important pro-angiogenic and immunosuppressive molecule critical for the development and growth of tumors.

Immature angiogenic vessels enable increased leakage of plasma proteins into tumor tissue. This in turn results in increased interstitial fluid content and lymph formation. Poor lymphatic drainage of solid tumors into the blood and tumor-draining lymph nodes leads to a buildup of fluid and thus high interstitial fluid pressure within the tumor (Reviewed in 310). High interstial fluid pressure forms a barrier to postcapillary transport, thereby reducing the ability of vascular drug delivery to the tumor (311, 312). Histochemical studies evaluating the morphological features of lymphatic vessels revealed that lymphatic vessel diameters were decreased and vessels were compressed within tumor tissues (313-315). In fact, tumor peripheral lymphatic vessels retain functionality, whereas intratumoral lymphatic vessels become compressed and nonfunctional due to rapid proliferation of tumor cells (316). Importantly, tumor secretion of VEGF can induce lymphangiogenesis within the tumor-draining lymph node, prior to lymph node metastasis (317, 318). Thus, while intratumor lymph drainage may

be poor, lymphangiogenesis of lymph nodes and peritumoral tissue may play a role in metastasis and immune activation in tumor-draining lymph nodes.

IX. Study Rationale:

L-selectin is constitutively expressed by all leukocytes and functions in lymphocyte migration to secondary lymphoid tissue through HEVs. L-selectin expression levels are similar between $CD4^+$ T cells and Treg cells, although L-selectin turnover is much higher on Treg cells. Importantly, L-selectin-mediated migration is required for optimal $CD4^+$ T cell and Treg cell entry into peripheral lymph nodes (82, 192). Evasion of immune responses by tumors is in part mediated by Treg cells through mechanisms of effector cell inhibition via cell-cell contact and cytokine-mediated suppression (43, 319). Therefore Treg cell accumulation at sites of anti-tumor immune responses is necessary for mediating their suppressive function. However, the role of L-selectin function in Treg cell entry into tissue or tissue-draining lymph nodes during chronic inflammation, such as cancer, has not been well described. Therefore, the overall goal of this study was to determine the role of L-selectin in mediating Treg migration to tumor and tumor-draining lymph nodes during tumor progression.

L-selectin can synergize with other adhesion molecules by activation of integrins through intercellular signaling pathways (124, 125, 320, 321). Furthermore, L-selectin signaling can enhance chemotaxis to SLC and inflammation-induced leukocyte chemotaxis *in vitro* (322, 323). Synergistic interactions between L-selectin and LFA-1/ICAM and $\alpha_4\beta_7$ integrin/MAdCAM-1 result in decreased lymphocyte rolling velocities, increased lymphocyte adhesion *in vitro* and direct subset-specific homing to secondary

lymphoid and non-lymphoid tissue *in vivo* (82, 119, 120, 127). However, L-selectin may also cooperate with other adhesion molecules to direct lymphocyte migration to sites of inflammation. Specifically, lymphocyte subsets that express $\alpha_4\beta_7$ integrin, such as Treg cells, may specifically home to inflamed endothelium by binding to VCAM-1 (110). However, it is not known if, under conditions of shear flow, capture of lymphocytes by selectins may be required for $\alpha_4\beta_7$ integrin/VCAM-1 interactions to occur. Therefore, in the present studies, the role of L-selectin function together with $\alpha_4\beta_7$ integrin/VCAM-1 interactions under shear flow was investigated. Under conditions of low shear, $\alpha_4\beta_7$ integrin/VCAM-1 interactions could induce adhesion of lymphocytes *in vitro*. Importantly, L-selectin function was required for $\alpha_4\beta_7$ integrin/VCAM-1-mediated lymphocyte adhesion under physiologic shear flow. Furthermore, Treg cell adhesion was significantly enhanced in the presence of both L-selectin function and $\alpha_4\beta_7$ integrin/VCAM-1 interactions at physiologic shear, compared to either one alone. Therefore, L-selectin cooperates with $\alpha_4\beta_7$ integrin/VCAM-1, and enhances $\alpha_4\beta_7$ integrin/VCAM-1-mediated Treg cell adhesion *in vitro*.

Since the 1980s, evidence for the role of Treg cells in tumor immune tolerance has been demonstrated in several models of cancer (324-326). While, anti-tumor immune responses are generated at the tumor site and within tumor-draining lymph nodes, Treg cells also accumulate within these same tissues and foster a suppressive environment for effector responses (327-329). The importance of Treg cells in tumor progression has been demonstrated by studies in mice in which depletion of total $CD4^+$ T cells induced tumor rejection and the *in vivo* antibody depletion of Treg cells resulted in antitumor effects (330). Therefore, we sought to determine the role of L-selectin in Treg cell accumulation

in tumor and tumor-draining lymph nodes during tumor progression using a 4T1 murine breast cancer model. During tumor progression, Treg cells accumulated preferentially in tumor-draining lymph nodes compared to contralateral non-draining lymph nodes, indicating a preferential homing to the tumor-draining lymph nodes. Importantly, Treg cells accumulated at a higher rate than $CD4^+$ T cells in lymph nodes during tumor progression. In addition, L-selectin deficiency severely reduced both $CD4^+$ T cell and Treg cell accumulation in tumor-draining lymph nodes and the tumor itself. Therefore, L-selectin is required for normal Treg distribution within secondary lymphoid tissue and tumor tissue during 4T1 tumor progression.

Lymphocyte accumulation within tumors and tumor-draining lymph nodes may be due to homing molecules that direct subset-specific migration. Lymphocyte migration depends on both attraction of cells through chemokine gradients and expression of adhesion molecule on the surface of both the leukocyte and endothelium. Specifically, Treg cells have been shown to preferentially migrate and accumulate in tumors and ascites; their migration is mediated by adhesion molecules in tumor vasculature and chemokines present in tumor microenvironments (266). In addition to chemokine receptors, L-selectin has been shown to be essential for Treg cell migration to PLN (192), but the role of L-selectin in Treg cell migration to tumor sites, surrounding tissue and tumor-draining lymph nodes is unknown.

The role of L-selectin in Treg cell migration was determined using adoptive transfer assays. Treg cell migration to lymph nodes occurred primarily through HEV in an L-selectin-dependent manner. However, in L-$selectin^{-/-}$ mice, some L-selectin-independent migration into lymph nodes was observed. Interestingly, Treg cell migration

into late stage tumor tissue was drastically reduced in L-selectin$^{-/-}$ mice. Thus, L-selectin is required for Treg cell migration into lymph nodes through HEV, and significantly contributes to Treg cell migration into tumor tissue.

These studies contribute significantly to characterizing the role of L-selectin in Treg cell interactions with inflamed endothelium. In addition, this work contributes to the understanding of L-selectin-mediated Treg cell migration during conditions of chronic inflammation such as cancer. The role of L-selectin in Treg cell migration to tissue-draining lymph nodes may provide innovative approaches to inducing Treg cell migration during chronic inflammatory conditions and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, and asthma. Furthermore, Treg accumulation in tumor-draining lymph nodes may be critical in tumor immune evasion, therefore, these studies provide insights that may contribute to the development of novel immunotherapeutic strategies.

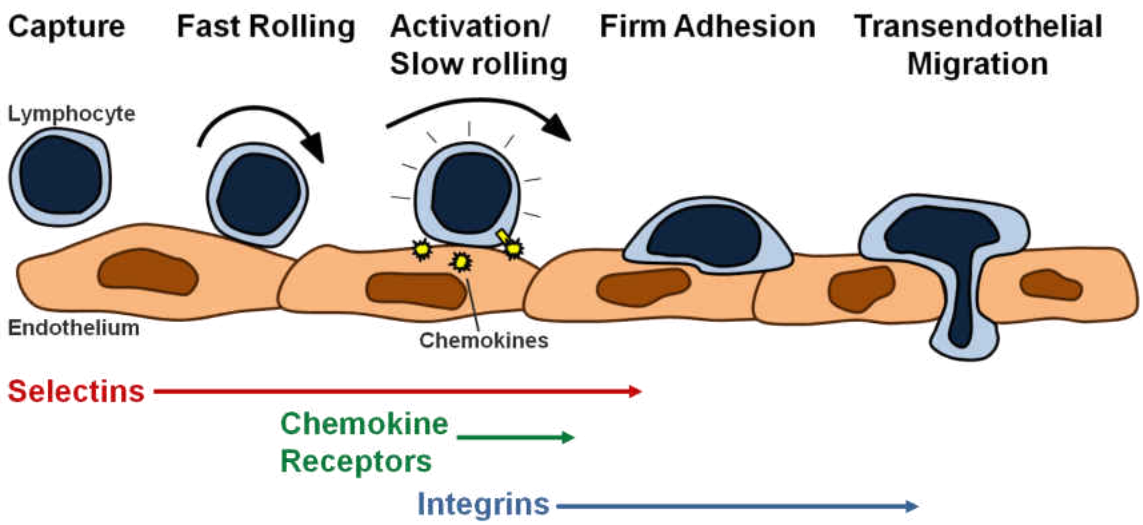


Figure 1. Leukocyte migration is mediated by a multistep adhesion cascade.

Initial adhesive interactions, termed capture and rolling, between leukocytes and endothelium are mediated by the selectin family of adhesion molecules. Upon capture, lymphocytes roll along endothelium at high velocities, and chemokine receptors on leukocytes are able to bind chemokines present on endothelial cells. Chemokine-mediated activation induces conformational changes of integrins to a state of high-affinity binding. Integrin binding to cell adhesion molecules facilitates slow rolling and firm adhesion of leukocytes to the endothelium. Upon firm adhesion, leukocytes transmigrate through the vessel wall and chemotax to their target site.

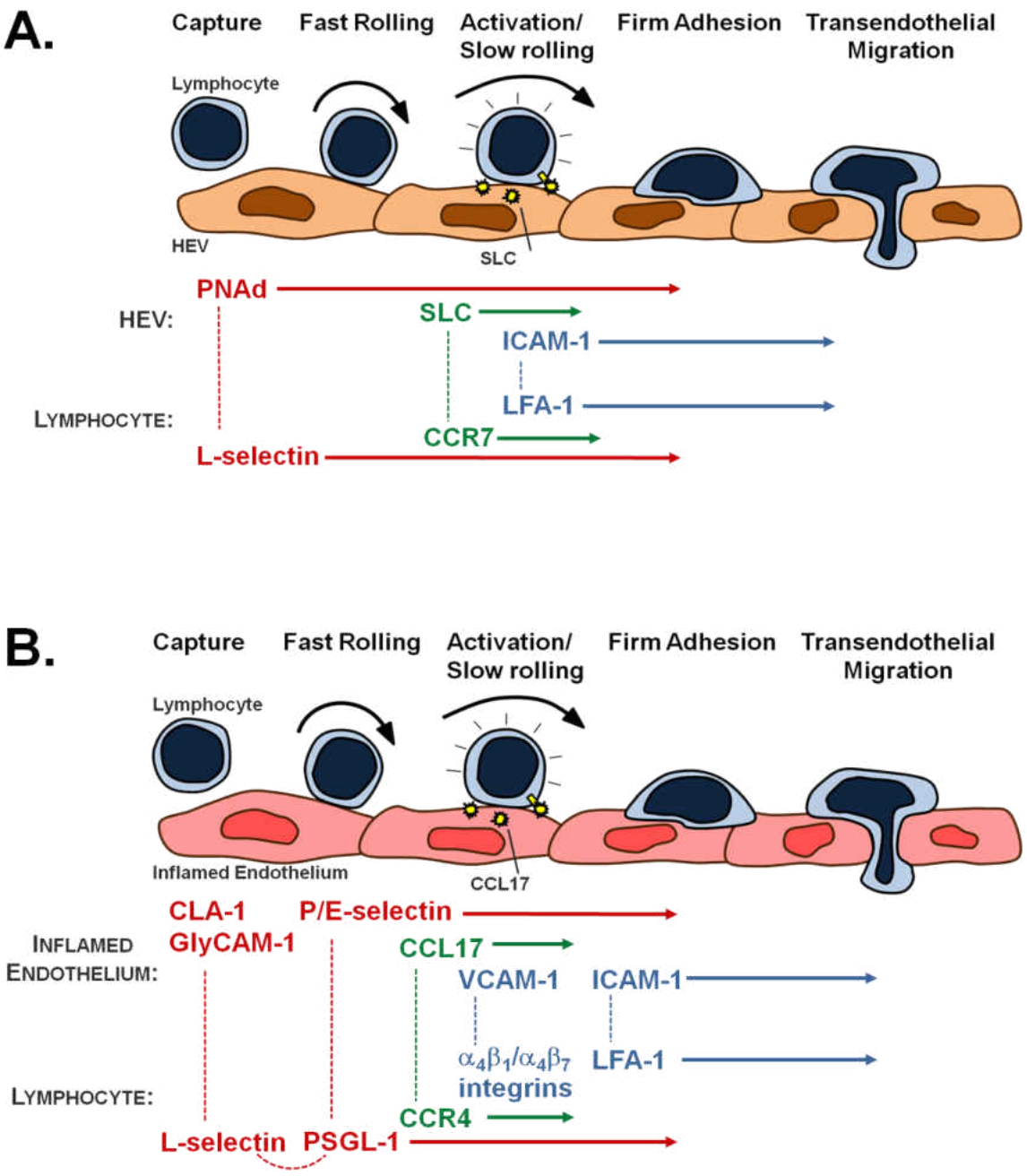


Figure 2. Lymphocyte migration through HEVs and to sites of cutaneous inflammation is mediated by a multistep adhesion cascade.

Lymphocyte migration into lymph nodes and sites of cutaneous inflammation is directed by specific adhesion molecules and chemokine/chemokine receptor interactions. A) Lymphocyte migration into peripheral lymph nodes is dependent on adhesion molecule

interactions with high endothelial venules (HEV). L-selectin-dependent lymphocyte capture and rolling enables lymphocyte expressed CC-chemokine receptor 7 (CCR7), to interact with secondary lymphoid-tissue chemokine (SLC) on HEV. Upon ligation of CCR7/SLC, lymphocyte function-associated antigen-1 (LFA-1) binds to intercellular cell adhesion molecule-1 (ICAM-1) on the endothelium and mediates firm adhesion. B) Lymphocyte migration to site of cutaneous inflammation is facilitated by the selectin family of adhesion molecules (L-, E-, P-selectin) binding to their respective ligands (eg. Cutaneous lymphocyte antigen-1, CLA-1; glycosylation-dependent cell adhesion molecule-1, GlyCAM-1; P-selectin glycoprotein ligand-1, PSGL-1) . Upon capture, lymphocyte activation is facilitated by CC-chemokine receptor (eg. CCR4) binding to chemokines (eg. CCL17) on vascular endothelium. Chemokine-mediated activation results in integrin conformational changes enabling binding of integrins (eg. $\alpha_4\beta_7$ integrin, $\alpha_4\beta_1$ integrin, or LFA-1) to vascular cell adhesion molecule-1 (VCAM-1) or ICAM-1 on endothelial cells, resulting in firm adhesion of lymphoytes and eventual transendothelial migration.

CHAPTER 2

L-SELECTIN FUNCTION FACILITATES $\alpha_4\beta_7$ INTEGRIN INTERACTIONS WITH VCAM-1 UNDER PHYSIOLOGIC SHEAR

ABSTRACT

Lymphocyte migration is regulated by a cascade of adhesion molecule interactions. Specifically, lymphocyte rolling is mediated by selectins, while slow rolling and adhesion are attributed to integrin and Ig family members. $\alpha_4\beta_7$ integrin interactions with its primary ligand, mucosal addressin cell adhesion molecule-1, synergize with L-selectin function to regulate the recirculation of naïve lymphocytes to gut-associated mucosal tissues. However, subsets of effector, memory, and regulatory T cells also express high levels of L-selectin and $\alpha_4\beta_7$ integrin, and may instead bind to vascular cell adhesion molecule-1 (VCAM-1), a secondary ligand for $\alpha_4\beta_7$ integrin, to support migration. Therefore, potential synergism between L-selectin function and $\alpha_4\beta_7$ integrin/VCAM-1 interactions were examined. To determine the ability of $\alpha_4\beta_7$ integrin to interact with VCAM-1 under conditions of shear, native and L-selectin-transfected TK-1 (TK-$1^{L\text{-}sel}$) T cell interactions with L-selectin ligand (fucosyltransferase VII)- and/or VCAM-1-transfected EA.hy926 endothelial cell lines were assessed using an *in vitro* flow chamber assay. While native TK-1 cells, expressing $\alpha_4\beta_7$ but not $\alpha_4\beta_1$ integrin, showed little interaction with VCAM-1-transfected endothelial cells, the addition of L-selectin function resulted in a six-fold increase in number of rolling cells. Furthermore, $\alpha_4\beta_7$ integrin/VCAM-1 interactions stabilized L-selectin-mediated rolling, as demonstrated by a 64% reduction in rolling velocity, and increased $\alpha_4\beta_7$ integrin/VCAM-1-mediated TK-$1^{L\text{-}sel}$ cell adhesion by ~30-fold at physiologic shear. Importantly, $\alpha_4\beta_7$ integrin/VCAM-1 interactions significantly contributed to regulatory T cell adhesion to transfected endothelial cells under shear stress only in the presence of L-selectin function.

Thus, functional synergy between L-selectin and $\alpha_4\beta_7$ integrin/VCAM-1 interactions may promote lymphocyte recruitment to sites of inflammation.

INTRODUCTION

Migration of leukocytes out of the blood into lymphoid and non-lymphoid tissues enables the immune system to elicit rapid and effective responses. The process of leukocyte migration from the blood stream and into secondary lymphoid tissues or sites of inflammation requires a complex series of steps to overcome hydrodynamic forces, or shear, of the rapidly moving blood. This process is intricately coordinated by various adhesion molecules and ligands expressed on the surface of both leukocytes and the endothelium in a process known as the "adhesion cascade" (331, 332). Initial capture of leukocytes is primarily mediated by the selectin family of adhesion molecules that binds to mucin family ligands causing the cells to tether and roll along the endothelium (61, 62).

L-selectin is constitutively expressed on all classes of leukocytes and plays an important role in mediating capture of leukocytes from the blood stream and also supports rolling on the vascular endothelium independent of E- or P-selectins (80, 82). Lymphocyte migration through high endothelial venules (HEV) into mucosal sites such as Peyer's patches and mesenteric lymph nodes (MLN) is primarily regulated by L-selectin and $\alpha_4\beta_7$ integrin interactions with their primary ligands, peripheral node addressin (PNAd) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), respectively (105, 127, 333). Vascular L-selectin ligand expression can also be induced on the endothelium of inflamed tissues, such as those found in cutaneous sites of chronic inflammation, acute dermatitis, rheumatoid arthritis, asthma, diabetes, Grave's disease and Hashimoto's thyroiditis (83). Specifically, cutaneous lymphocyte-associated antigen-1 (CLA-1) serves as a L-selectin ligand on inflamed endothelium and can be identified by

the human endothelial cell antigen-452 (HECA-452) mAb (334). In addition, leukocytes expressing L-selectin can bind to other leukocytes or microparticles attached to the endothelium in a P-selectin glycoprotein ligand-1 (PSGL-1)-dependent manner in a process known as secondary tethering that has been shown to increase leukocyte recruitment at sites of inflammation (335-337).

Integrins also play an important role in leukocyte migration during both recirculation and inflammation. $\alpha_4\beta_7$ integrin is expressed by most lymphocytes and supports migration to mucosal-associated lymphoid tissues including the Peyer's patches and MLN through binding to MAdCAM-1 (101, 102, 338). During late stages of inflammation, recruitment can be mediated by a well-characterized high affinity interaction between leukocyte-expressed very late antigen-4 (VLA-4, $\alpha_4\beta_1$ integrin) and vascular cell adhesion molecule-1 (VCAM-1) expressed on the inflamed endothelium, which is able to mediate both capture and rapid adhesion (106, 173, 222). Importantly, $\alpha_4\beta_7$ integrin binding to VCAM-1 has also been demonstrated, but this interaction has not been well defined (106-109). Interestingly, it has recently been shown that $\alpha_4\beta_7$ integrin-mediated binding to VCAM-1 was essential for the migration of T cells to sites of cutaneous inflammation during skin contact hypersensitivity responses (110). However, it is unknown whether $\alpha_4\beta_7$ integrin/VCAM-1 interactions require the presence of other adhesion molecules, specifically those that function early in the adhesion cascade, to facilitate capture and high velocity rolling of lymphocytes.

Optimal function of integrins during leukocyte recruitment occurs after the cells have been captured and their velocity reduced (339) by selectin-mediated interactions. Subsequent integrin-ligand interactions facilitate slower rolling velocities and eventually

firm arrest of the rolling cell (72, 73). Previous studies have demonstrated cooperation of L-selectin/ligand binding together with lymphocyte function associated antigen-1 (LFA-1) binding with intercellular cell adhesion molecule-1 (ICAM-1) to enhance leukocyte recruitment to sites of inflammation by stabilizing leukocyte/endothelial cell interactions and decreasing leukocyte rolling velocities (119, 120, 124, 126). Synergistic interactions have also been demonstrated between L-selectin and $\alpha_4\beta_7$ integrin in the presence of MAdCAM-1; however, it is unknown whether this synergism can occur at sites of inflammation, where VCAM-1, but not MAdCAM-1, is expressed (127, 128). Importantly, L-selectin and $\alpha_4\beta_7$ integrin are coexpressed by multiple populations of lymphocytes, including naïve T and B cells, regulatory T cells (Treg), and subsets of memory/effector T and B cells (198, 199, 333, 340-344). Treg cells remain the focus of intense study due to the essential role they play in regulating immune homeostasis and maintenance of immunological self-tolerance through suppression of pathological and physiological immune responses (2-4). Migration of Treg cells to sites of inflammation is critically important to their function since secretion of cytokines and cell-cell contact of Treg cells with effector T cells and antigen presenting cells (APC) is necessary for Treg cells to exert their suppressive effects (189).

Therefore, to assess the cooperation between L-selectin and $\alpha_4\beta_7$ integrin under conditions of shear flow, the well characterized endothelial cell line, EA.hy926, was used in an *in vitro* flow chamber assay system (126, 334, 345). To determine if $\alpha_4\beta_7$ integrin interactions with VCAM-1 synergize with L-selectin function, endothelial cell lines expressing L-selectin ligand and/or VCAM-1 were used in conjunction with the $\alpha_4\beta_7$ integrin-expressing TK-1 cell line transfected to express L-selectin. Furthermore, to

demonstrate a synergistic role for L-selectin and $\alpha_4\beta_7$ integrin in murine Treg cell adhesion under shear stress, interactions between endothelial cell lines and lymphocytes from forkhead box p3 (Foxp3)^{EGFP} mice were assessed.

Consistent with previous studies, L-selectin-mediated rolling was shear dependent, while the velocity of rolling TK-1 cells was shear independent. $\alpha_4\beta_7$ integrin interactions with VCAM-1 occurred only in conditions of low shear, resulting in slow rolling velocities and firm adhesion of TK-1 cells to VCAM-1 expressing monolayers. In addition, velocity of TK-1$^{L\text{-}sel}$ rolling cells was reduced at physiologic shear in the presence of $\alpha_4\beta_7$ integrin/VCAM-1 interactions. These results showed that $\alpha_4\beta_7$ integrin interactions with VCAM-1 stabilized L-selectin-dependent rolling and promoted adhesion at physiologic shear. The results of this study demonstrate functional synergy between L-selectin and $\alpha_4\beta_7$ integrin/VCAM-1 interactions under shear flow. Therefore, synergistic interactions between L-selectin and $\alpha_4\beta_7$ integrin/VCAM-1 interactions may play a role in recruitment of lymphocyte subsets, such as Treg cells, to sites of inflammation.

MATERIALS AND METHODS

Mice

Foxp3^{EGFP} mice (346) on the Balb/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed and bred in a specific pathogen-free barrier facility and screened regularly for pathogens. Mice were 7-12 weeks old for all experiments. All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin – Milwaukee.

Murine Lymphocytes

Lymphocytes were harvested from inguinal, axillary, brachial, and cervical lymph nodes of Foxp3^{EGFP} mice and single-cell suspensions were prepared as previously described (125). Peripheral lymph node (PLN) cells ($25x10^6$/ml) were treated with 20 μg/ml of either control Ab (rat IgG) or $\alpha_4\beta_7$ integrin blocking antibody (DATK32; ATCC, Manassas, VA), purified by protein G affinity chromatography, and allowed to incubate at 37°C for 15 min before *in vitro* rolling and adhesion assays were performed. PLN cells were also labeled for adhesion molecule expression and analyzed by flow cytometry as described below.

Cells and Cell lines

A murine T cell lymphoma cell line, TK-1 (CRL-2396, ATCC) was transfected with human L-selectin cDNA (347) using the pcDNA™ 3.1 expression vector (Invitrogen, Gaithersburg, MD) to generate the TK-1$^{L\text{-}sel}$ cell line. TK-1 cell lines were maintained in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, all from Invitrogen). Transfected TK-1 cell lines were maintained in complete RPMI 1640

medium containing G-418 (1 mg/ml, Mediatech, Manassas, VA). L-selectin, $\alpha_4\beta_1$ integrin, and $\alpha_4\beta_7$ integrin expression levels on Foxp3^{EGFP} lymphocytes, TK-1, and TK-1$^{L\text{-}sel}$ cell lines were determined by staining with the following purified, biotinylated-, phycoerythrin- (PE), fluorescein isothiocyanate- (FITC), allophycocyanin-, or PE/Cy7-conjugated antibodies: anti-L-selectin (LAM1-116, Ref. 125), anti-mouse β_1 integrin (CD29, HMβ1-1 Biolegend, San Diego, CA), anti-mouse α_4 integrin (CD49d, R1-2; Biolegend), anti-β_7 integrin (M293), or anti-$\alpha_4\beta_7$ integrin (DATK32; both BD Biosciences, San Jose, CA) mAbs. Biotinylated antibody staining was revealed using PE-conjugated neutralite-avidin (Southern Biotech, Birmingham, AL), and purified antibody was detected using PE-conjugated goat anti-rat IgG F(ab')$_2$ (eBioscience, San Diego, CA). Isotype matched mouse IgG Abs (Southern Biotech) were used as controls for background staining in all experiments. Data was collected using a FACSCalibur flow cytometer using CellQuest™ Pro software (BD Biosciences). Cells with light scatter properties of mononuclear cells were gated and a minimum of 10,000 events were collected per experiment. Lymphocytes were further analyzed for expression levels of cell surface molecules using FlowJo analysis software (Tree Star Inc., Ashland, OR). Treg cells were identified by EGFP expression and a minimum of 10,000 EGFP$^+$ cells were collected for each experiment.

The native EA.hy926 (926) cells were a gift from Dr. C.-J. Edgell (348). 926 cells expressing α1,3-fucosyltransferase (926-FtVII) were previously described (334). 926-VCAM-1 cells were generated by transfection of 926 cells with human VCAM-1 cDNA (gift from Dr. D. Staunton, ICOS Pharmaceuticals, Bothell, WA) using the pcDNA™ 3.1 expression vector. 926-FtVII/VCAM-1 cells were generated by cotransfection of 926

cells with FtVII and VCAM-1 cDNAs. Transfectants were selected with G418 and clones of 926-VCAM-1 and 926-FtVII/VCAM-1 cells were obtained by limiting dilution and sorting by flow cytometry (FACStar, BD Biosciences, Mountain View, CA). Prior to sorting, cells were stained for the HECA-452 epitope and/or VCAM-1 using purified anti-human CLA-1 (BD Biosciences) mAb and detected using secondary goat anti-rat IgM-FITC (Southern Biotech), and PE-conjugated anti-human CD106 (51-10C9, BD Biosciences) mAb, respectively, as previously described (126). All 926 cell lines were maintained in complete DMEM medium (DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin). Transfected 926 cell lines were maintained in complete DMEM medium containing G-418 (1 mg/ml). Expression levels of cell surface molecules were analyzed on a FACSCalibur flow cytometer (BD Biosciences) after staining as above. Transfected 926 cell lines with similar expression levels of the HECA-452 epitope and VCAM-1 were used for all experiments.

Rolling and adhesion under defined flow conditions

An *in vitro* flow chamber assay was used to assess the interactions of TK-1 cells or murine lymphocytes with 926 cell lines under flow conditions. The 926 cell lines were grown to confluence in 35 mm^2 culture dishes (Corning, Corning, NY) and assembled in a parallel-plate flow chamber per the manufacturer's instructions (circular flow chamber kit, Glycotech, Gaithersburg, MA) to produce a uniform laminar flow field (349). Lymphocytes ($1x10^6$/ml) were suspended in flow buffer (phosphate buffered saline containing 0.75 mM $CaCl_2$, 0.75 mM $MgCl_2$, and 0.5% (w/v) bovine serum albumin) and drawn over confluent monolayers of endothelial cell lines at defined shear stresses using

a programmable syringe pump (Harvard Apparatus, Natick, MA). Lymphocyte-endothelial cell interactions were observed by phase contrast and fluorescent microscopy on an inverted Nikon TE2000-U microscope (Nikon, Melville, NY) and recorded using a CoolSNAP ES digital monochromatic CCD camera (Photometrics, Tucson, AZ). Digital video acquisition was performed using MetaVue™ software (Universal Imaging Corp., Downington, PA) at a rate of 30 frames/second using 10X (Nikon Plan Fluor) or 20X (Nikon Plan Fluor ELWD) objectives. Lymphocyte-endothelial cell interactions were analyzed by video play-back using a digital time stamp. Lymphocyte rolling was determined by counting the number of cells that crossed a 665 μm wide field over a 10 s time period. In experiments in which shear change occurred, flow was initiated at 2.5 dyne/cm^2 and reduced step-wise in 2 min intervals with a 20 s period in between recording to allow for stabilization of flow. For each experiment, the mean rolling velocity of from 20-100 cells was determined, with lower numbers of cells being analyzed due to fewer interactions with the endothelium at low or high shear stresses or due to rapid adherence without rolling. Rolling velocities of cells were calculated by dividing the distance each cell traveled between two points by the elapsed time. During each experiment, a minimum of three videos ranging from 15-45 s in random fields were analyzed and averaged. For adhesion assays, cells were drawn over the 926 cell lines for 10 min at low (0.25 dyne/cm^2), intermediate (1.0 dyne/cm^2) or physiologic (1.75 dyne/cm^2) shear stress, unbound cells were washed away using flow buffer and still images were acquired from a minimum of three random fields. The total number of cells, and in some cases the number of EGFP$^+$ cells, bound/0.6x10^6 μm^2 area was determined for each experimental condition.

Statistical analysis

All data are presented as mean values ± SEM. Significant differences between sample means were determined using one-way ANOVA and the Student's *t* test. A p value of <0.05 was considered to be statistically significant.

RESULTS

In vitro flow chamber assay

The contribution of L-selectin and $\alpha_4\beta_7$ integrin to lymphocyte rolling and adhesion was determined by analyzing lymphocyte interactions with cultured endothelial cell monolayers under defined shear stress using a parallel plate flow chamber assay. EA.hy926 monolayers were grown to confluence on 35 mm^2 dishes and assembled in a flow chamber consisting of an acrylic base with a port for fluid entry, exit, and vacuum. The acrylic base was assembled on a 12.7 μm rubber gasket to create a vacuum seal with an opening of 2.0 cm in length and 2.5 mm in width for viewing of cell-cell interactions (Fig. 3A). Lymphocytes were withdrawn through the flow chamber at calculated shear stresses by a programmable syringe pump (Fig. 3B). Lymphocyte-endothelial interactions were recorded using a high-speed digital camera, and analyzed using Metaview™ software (Fig. 3C).

Cell line adhesion molecule expression

Native unactivated 926 cells have served as a useful model system for measuring the contributions of individual adhesion molecules to leukocyte rolling and adhesion due to negligible expression of most vascular adhesion molecules including P-selectin, E-selectin, ICAM-1, CD34, PSGL-1, VCAM-1, and vascular adhesion protein-1 (126, 334, 345). Previously, 926-FtVII cells were shown to express functional L-selectin ligand that can be identified by staining for the HECA-452 epitope (126). To examine $\alpha_4\beta_7$ integrin/VCAM-1 interactions under shear flow, transfected 926 cell lines that expressed VCAM-1 were generated. In addition, 926 cells were co-transfected with FtVII and VCAM-1 cDNA to further elucidate a role for L-selectin function in mediating

$\alpha_4\beta_7$ integrin/VCAM-1 interactions. Native 926 cells showed no HECA-452 or anti-VCAM-1 mAb reactivity, while both 926-FtVII and 926-FtVII/VCAM-1 cells showed equivalent expression levels of the HECA-452 epitope (Fig. 4). Similarly, both 926-VCAM-1 and 926-FtVII/VCAM-1 transfected cell lines showed equivalent expression levels of VCAM-1.

L-selectin/ligand and $\alpha_4\beta_7$ integrin/VCAM-1 interactions are difficult to study in many experimental model systems due to the presence of VLA-4. To determine $\alpha_4\beta_7$ integrin interactions alone with VCAM-1, the murine TK-1 T cell line that constitutively expresses high levels of $\alpha_4\beta_7$ integrin but does not express VLA-4 was used (70, 107). To assess the contribution of L-selectin and $\alpha_4\beta_7$ integrin to leukocyte rolling and adhesion, the L-selectin expressing TK-$1^{L\text{-}sel}$ transfected cell line was used. Native TK-1 cells showed no L-selectin expression, while TK-$1^{L\text{-}sel}$ cells showed high levels of L-selectin expression (Fig. 5). Staining with anti-$\alpha_4\beta_7$ integrin mAb showed similarly high levels of $\alpha_4\beta_7$ integrin expression in native TK-1 and TK-$1^{L\text{-}sel}$ cell lines (Fig. 5). Thus $\alpha_4\beta_7$ integrin interactions with VCAM-1 could be assessed in the absence or presence of L-selectin function.

TK-1 cell rolling in the presence of L-selectin ligand and VCAM-1

The contribution of L-selectin and $\alpha_4\beta_7$ integrin to lymphocyte rolling was determined by analyzing TK-1 and TK-$1^{L\text{-}sel}$ cell interactions with 926 cell monolayers under defined shear stress using a parallel plate flow chamber assay. Untransfected TK-1 cells did not roll on native 926, or 926-FtVII monolayers at any shear stress examined (Fig. 6). In addition, untransfected TK-1 cells did not interact with 926-VCAM-1 or 926-FtVII/VCAM-1 monolayers at higher shear stresses (1.25-2.5 dyne/cm^2), but did show

minimal rolling at lower shear stresses (0.25-1.0 dyne/cm^2). Thus, $\alpha_4\beta_7$ integrin alone showed little ability to interact with VCAM-1 in this system.

As expected, native 926 monolayers did not support TK-1$^{L\text{-}sel}$ cell rolling at any shear stress examined (Fig. 6). Compared to native TK-1 cells, TK-1$^{L\text{-}sel}$ cells showed significantly increased rolling on 926-FtVII monolayers at each shear stress examined ($p<0.001$), as previously reported for other L-selectin-expressing cell types (126, 334, 350). By contrast, TK-1$^{L\text{-}sel}$ cells did not show an increased ability over that of TK-1 cells to interact with 926-VCAM-1 monolayers.

In general, TK-1$^{L\text{-}sel}$ cell rolling on 926-FtVII monolayers was significantly increased compared to that observed on 926, 926-VCAM-1, or 926-FtVII/VCAM-1 monolayers ($p<0.05$). In fact, rolling of TK-1$^{L\text{-}sel}$ cells was significantly reduced (~50-90%; $p<0.05$) on 926-FtVII/VCAM-1 monolayers compared to that on 926-FtVII monolayers at all shear stresses examined. Despite this, rolling was still increased by ~75-99% ($p<0.05$) over that observed on 926 or 926-VCAM-1 monolayers. Thus, these results demonstrate that $\alpha_4\beta_7$ integrin/VCAM-1 interactions alone occurred minimally and only under conditions of low shear stress but this interaction significantly affected L-selectin-dependent rolling across shear stresses.

$\alpha_4\beta_7$ integrin/VCAM-1 interactions mediate slower L-selectin-dependent rolling velocities

To determine whether $\alpha_4\beta_7$ integrin interactions with VCAM-1 stabilized L-selectin-mediated rolling interactions, rolling velocity was analyzed under conditions of low (0.25 dyne/cm^2) and physiologic (1.75 dyne/cm^2) shear stress. Rolling velocities

of TK-1$^{L\text{-}sel}$ cells were determined by quantifying the distance a cell rolled over a defined period of time using a digital time stamp (Fig 7A). TK-1$^{L\text{-}sel}$ cells rolled on 926-FtVII monolayers at median velocity of 132 μm/sec at 1.75 dynes/cm^2 which is similar to what has been previously reported for other L-selectin-expressing cells (Fig. 7B, Ref. 126). The addition of VCAM-1 (926-FtVII/VCAM-1) resulted in a dramatic shift to the left of the velocity curve (median velocity of 8 μm/s). Interestingly, while the majority of the TK-1$^{L\text{-}sel}$ cells exhibited slow rolling on 926-FtVII/VCAM-1 monolayers, some cells rolled at intermediate velocities while others rolled at characteristic L-selectin-mediated velocities (Fig. 7B). Only rare TK-1$^{L\text{-}sel}$ cells were observed to interact with 926-VCAM-1 monolayers at physiologic shear (<100 cells from all experiments). However, under low shear stress, the few TK-1$^{L\text{-}sel}$ cells that were observed to roll on 926-VCAM-1 monolayers did so at extremely slow velocity (median velocity of 2 μm/sec; Fig. 7B). Compared to physiologic shear stress, TK-1$^{L\text{-}sel}$ cell rolling velocities on 926-FtVII monolayers at low shear stress were similar, confirming L-selectin-mediated rolling velocity is shear stress independent (Fig. 7C). By contrast, TK-1$^{L\text{-}sel}$ cell rolling velocities on 926-FtVII/VCAM-1 monolayers tended to be increased at physiologic shear compared to low shear. Taken together, these results show $\alpha_4\beta_7$ integrin/VCAM-1 interactions, at physiologic shear stress, stabilized L-selectin-mediated rolling.

TK-1$^{L\text{-}sel}$ cell adhesion is augmented by both L-selectin ligand and VCAM-1 expression

The contribution of both L-selectin/ligand and $\alpha_4\beta_7$ integrin/VCAM-1 interactions to TK-1 cell adhesion was determined using flow assays at low, intermediate,

and physiologic shear stress (0.25, 1.0, and 1.75 dyne/cm^2, respectively) for a 10 min time period. Neither 926 nor 926-FtVII monolayers supported adhesion of TK-1 or TK-1$^{L\text{-}sel}$ cells at any shear stress examined (data not shown). By contrast, 926-VCAM-1 monolayers were able to support equivalent TK-1 and TK-1$^{L\text{-}sel}$ cell adhesion at low shear stress, (Fig. 8A, C). At intermediate shear stress, adhesion of both cell lines to 926-VCAM-1 monolayers was significantly reduced compared to low shear (by ~70%, $p<0.05$). Furthermore, at physiologic shear stress, $\alpha_4\beta_7$ integrin interactions with VCAM-1 were unable to mediate TK-1 or TK-1$^{L\text{-}sel}$ cell adhesion to 926-VCAM-1 monolayers as indicated by a 99% reduction ($p<0.01$) compared to adhesion at low shear (Fig 8A, C).

TK-1 and TK-1$^{L\text{-}sel}$ cells also adhered to 926-FtVII/VCAM-1 monolayers in similar numbers at low shear stress (Fig. 8B, C). Importantly, at intermediate shear stress, TK-1$^{L\text{-}sel}$ cells still showed the same high level of adhesion while TK-1 cell adhesion was reduced by ~7-fold compared to adhesion under low shear stress ($p<0.01$). The requirement for L-selectin function in $\alpha_4\beta_7$ integrin/VCAM-1-mediated adhesion was most strikingly demonstrated by an ~96% reduction in TK-1 cell adhesion compared to TK-1$^{L\text{-}sel}$ cell adhesion at physiologic shear stress (Fig. 8B, C, $p<0.001$). While the number of TK-1$^{L\text{-}sel}$ adherent cells also modestly decreased from intermediate to physiologic shear stress, this decrease was not statistically significant. Taken together, these results demonstrate that $\alpha_4\beta_7$ integrin interactions with VCAM-1 can support leukocyte adhesion at physiologic shear stress when L-selectin function is present. Furthermore, while $\alpha_4\beta_7$ integrin/VCAM-1 interactions can support adhesion at intermediate shear stress, adhesion is dramatically more efficient in the presence of L-selectin function.

Treg cells coexpress L-selectin and $\alpha_4\beta_7$ integrin

The coexpression of L-selectin and $\alpha_4\beta_7$ integrin by Treg cells isolated from PLNs of Foxp3^{EGFP} mice was determined using anti-L-selectin and anti-$\alpha_4\beta_7$ integrin mAbs and quantified by flow cytometry. Foxp3^{EGFP+} cells were gated (Fig. 9 *left*) and analyzed for adhesion molecule expression. Results showed that 72 ± 8% of the Treg cells expressed L-selectin while 30% ± 1% expressed $\alpha_4\beta_7$ integrin (n=3 independent experiments). Importantly, 27% ± 2% of the Treg cells coexpressed L-selectin and $\alpha_4\beta_7$ integrin (Fig. 9 *right*).

Treg cell rolling is independent of $\alpha_4\beta_7$ integrin function.

The contribution of L-selectin and $\alpha_4\beta_7$ integrin to Treg cell rolling under conditions of shear stress was assessed. Single-cell suspensions of lymphocytes isolated from PLNs of Foxp3^{EGFP} mice were treated with control or $\alpha_4\beta_7$ integrin blocking antibodies. Cells were then withdrawn over 926, 926-FtVII, -VCAM-1, or FtVII/VCAM-1 monolayers at physiologic shear stress (1.75 dyne/cm^2) and the number of rolling cells was determined using phase contrast or fluorescence microscopy. As expected, cells did not roll in the absence of L-selectin ligand on 926 and 926-VCAM-1 monolayers (Fig. 10). By contrast, in the presence of L-selectin ligand, significantly more cells rolled on 926-FtVII and 926-FtVII/VCAM-1 monolayers ($p < 0.05$). Treatment with $\alpha_4\beta_7$ integrin blocking antibody had no effect on number of total or EGFP$^+$ rolling cells at physiologic shear stress.

$\alpha_4\beta_7$ integrin mediates Treg cell adhesion to VCAM-1 under physiologic shear stress in the presence of L-selectin

The contribution of L-selectin and $\alpha_4\beta_7$ integrin to Treg cell adhesion on VCAM-1 under shear flow was assessed using PLN lymphocytes isolated from Foxp3^{EGFP} mice. Lymphocyte adhesion to transfected 926 monolayers was analyzed as above with Treg cells being identified by EGFP fluorescence. Both Treg and non-Treg lymphocytes showed little adhesion to native 926, 926-FTVII, or 926-VCAM-1 cell lines at physiologic shear stress (Fig. 11). However, adhesion to 926-FTVII/VCAM-1 monolayers was increased by 5.9-fold ($p<0.01$) for EGFP$^+$ Treg cells and 3.9-fold ($p<0.05$) for EGFP$^-$ cells compared to adhesion to 926-VCAM-1 monolayers. Since some Treg cells express $\alpha_4\beta_1$ integrin, cells were treated with 20 μg/ml $\alpha_4\beta_7$ integrin blocking or rat IgG control antibodies prior to the adhesion assay to demonstrate the role of $\alpha_4\beta_7$ integrin in lymphocyte adhesion. Blocking $\alpha_4\beta_7$ integrin significantly reduced both EGFP$^-$ lymphocyte and EGFP$^+$ Treg cell adhesion to 926-FTVII/VCAM-1 monolayers by 50% compared to control antibody-treated cells (Fig. 11). These results are consistent with $\alpha_4\beta_7$ integrin/VCAM-1-mediated adhesion in the presence of L-selectin function as demonstrated in the TK-1 adhesion assays. Taken together, these results demonstrate synergistic interactions between L-selectin and $\alpha_4\beta_7$ integrin/VCAM-1 function, and these interactions may play a role in recruitment of lymphocytes, such as Treg cells, to sites of inflammation.

DISCUSSION

The contribution of $\alpha_4\beta_7$ integrin/VCAM-1 interactions to lymphocyte rolling velocity and adhesion in the presence of functional L-selectin interactions was determined using a parallel plate flow chamber assay. In this study, transfected 926 endothelial cell lines expressing L-selectin ligand, VCAM-1, or both were used in conjunction with L-selectin-transfected TK-1 T cells expressing both $\alpha_4ß_7$ integrin and physiologic levels of L-selectin (Fig. 4, 5 and data not shown). Importantly, native TK-1 cells constitutively express high levels of $\alpha_4\beta_7$ integrin, but do not express either L-selectin or VLA-4. Under physiologic shear stress, only L-selectin-mediated interactions were able to support rolling (Fig. 6). However, under low shear stress where $\alpha_4ß_7$ integrin/VCAM-1 interactions can occur, both native TK-1 and TK-$1^{L\text{-}sel}$ cells interacted with 926-VCAM-1 monolayers. Notably, the frequency of rolling TK-$1^{L\text{-}sel}$ cells on 926-FtVII/VCAM-1 monolayers was significantly reduced from that observed on 926-FtVII monolayers (Fig. 6). This reduction in rolling frequency was not due to a lack of TK-$1^{L\text{-}sel}$ cell interactions with 926-FtVII/VCAM-1 cells, but rather to an increased rapid transition from rolling to firm adhesion (Fig. 8). In fact, in the presence of both L-selectin/ligand and $\alpha_4\beta_7$ integrin/VCAM-1, a majority of TK-$1^{L\text{-}sel}$ cells captured and adhered to the monolayer with only minimal slow rolling being observed (Fig. 7). Furthermore, these interactions were also found to apply to freshly isolated primary lymphocytes. Specifically, synergy between L-selectin function and $\alpha_4\beta_7$ integrin/VCAM-1 interactions were required for optimal Treg cell adhesion to occur under physiologic shear stress (Fig. 11). Taken together, these results demonstrate a functional synergy

between L-selectin and $\alpha_4\beta_7$ integrin/VCAM-1 interactions that may play a role in lymphocyte subset recruitment to sites of inflammation.

The results of this study demonstrate that in the absence of L-selectin function, $\alpha_4\beta_7$ integrin/VCAM-1 interactions were only able to initiate capture, albeit inefficiently, of TK-1 cells at reduced shear stresses. Physiologically, at sites of inflammation, normal flow rates are often disrupted and shear stress can be greatly decreased, allowing for integrin/ligand interactions to occur in the absence of selectin function (213, 351). Furthermore, interactions between $\alpha_4\beta_7$ integrins and VCAM-1 resulted in immediate firm arrest with little to no rolling (Figs. 6 and 8). The observed interactions of TK-1 cell lines with 926 cell lines expressing VCAM-1 are consistent with previous studies that characterized interactions between $\alpha_4\beta_1$ integrins and VCAM-1 in which lymphocytes initiated capture only at low shear stresses and lymphocyte interactions with VCAM-1 did not result in rolling, but underwent immediate firm arrest (71, 222). The present studies demonstrate that in the presence of L-selectin/ligand and $\alpha_4\beta_7$ integrin/VCAM-1 interactions, TK-1 cells were able to initiate capture and transition to immediate arrest at both intermediate and physiologic shear stresses.

Resting endothelial cells express very low levels of VCAM-1. By contrast, inflamed endothelium can be induced to express high levels of VCAM-1 by cytokines such as IL-1β, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α, 170, 352). VCAM-1 expression has been shown to play an important role in the recruitment of leukocytes to sites of acute and chronic inflammation in conditions such as arthritis, systemic lupus erythematosus, cancer, and asthma (353-356). While much research has focused on the role of VLA-4 interactions with VCAM-1 during inflammation, far fewer

studies have examined a role for $\alpha_4\beta_7$ integrin (107, 357, 358). However, VCAM-1 can also serve as a ligand for $\alpha_4\beta_7$ integrin in lymphocyte recruitment to cutaneous inflammation. In fact, a recent study by Ohmatsu et al. reported that lymphocyte Th1 effector cell migration during delayed-type hypersensitivity responses in the skin was inhibited in β_7 integrin-deficient mice, suggesting that $\alpha_4\beta_7$ integrin may direct lymphocyte homing during cutaneous inflammation (110). In addition to VCAM-1, expression of L-selectin ligands on inflamed endothelium has also been demonstrated. Specifically, CLA-1 has been shown to be upregulated on inflamed endothelium (350, 359), and L-selectin ligands are expressed during chronic inflammation in a wide variety of inflammatory diseases (Reviewed in 10). In addition, leukocyte-leukocyte interactions, in a process known as "secondary tethering", can occur at sites of inflammation by L-selectin ligation with PSGL-1 expressed on other leukocytes (125, 337). Therefore, under certain inflammatory conditions lymphocyte populations that continue to coexpress L-selectin and $\alpha_4\beta_7$ integrin may utilize this functional interaction to optimize migration.

The present studies also suggest a role for synergistic interactions between L-selectin and $\alpha_4\beta_7$ integrin/VCAM-1 in directing the efficiency and preferential homing of Treg subsets into peripheral tissues. While Treg cells demonstrate typical L-selectin-dependent migration to PLN, albeit less efficiently than their conventional $CD4^+$ T cell counterparts (192), they are also present in non-lymphoid sites including autoimmune lesions, cutaneous sites of inflammation, and tumors (196). Adhesion molecule expression has been characterized for subsets of Treg cells and shows that the predominant population of $CD4^+CD25^+Foxp3^+$ Treg cells express high levels of L-selectin (192, 197). This was also true in the present study where the majority of

Foxp3^{EGFP+} Treg cells expressed high levels of L-selectin (76%, Fig. 9). In addition to L-selectin, Treg cells display other adhesion molecules in diverse phenotypic patterns that divide them into complex subsets based on differential expression of these molecules. Specifically, Treg cells have been reported to have high $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrin expression (198). However, a study on human Treg cells showed that this expression pattern was not from a single population, but rather from two distinct populations of Treg cells expressing high levels of either $\alpha_4\beta_1$ or $\alpha_4\beta_7$ integrin (199). In the present study, the $\alpha_4\beta_7$ integrin was the predominant α_4 integrin expressed by murine Treg cells isolated from PLNs. Thus, while some information on the expression patterns of adhesion molecules for Treg cells exists, little is known about the individual or collective contributions of these adhesion molecules to the migration of Treg cells. In the current study, Treg cell adhesion mediated by $\alpha_4\beta_7$ integrin/VCAM-1 interactions was significantly increased in the presence of L-selectin function (Fig. 11). Furthermore, blocking $\alpha_4\beta_7$ integrin function with neutralizing antibody significantly reduced Treg cell adhesion, demonstrating that much of the observed adhesion could not be attributed to $\alpha_4\beta_1$ integrin-mediated binding to VCAM-1. These results show that cooperative interactions between L-selectin function and $\alpha_4\beta_7$ integrin/VCAM-1 can occur in primary Treg cell populations, and have the potential to direct their recruitment to sites of inflammation, where VCAM-1 is expressed. Further studies investigating the recruitment of Treg cell populations, or other effector lymphocyte populations, coexpressing L-selectin and $\alpha_4\beta_7$ integrin during inflammation need to be performed to demonstrate the physiologic importance of these interactions. Here we expand the repertoire of molecules that synergize with L-selectin to include $\alpha_4\beta_7$ integrin/VCAM-1 interactions and suggest

a novel role for these interactions at sites of inflammation. Taken together, these results may lead to a better understanding of population-specific recruitment, such as recruitment of Treg cells, to sites of inflammation. Ultimately, this may lead to improved therapeutic strategies against acute and chronic inflammatory conditions.

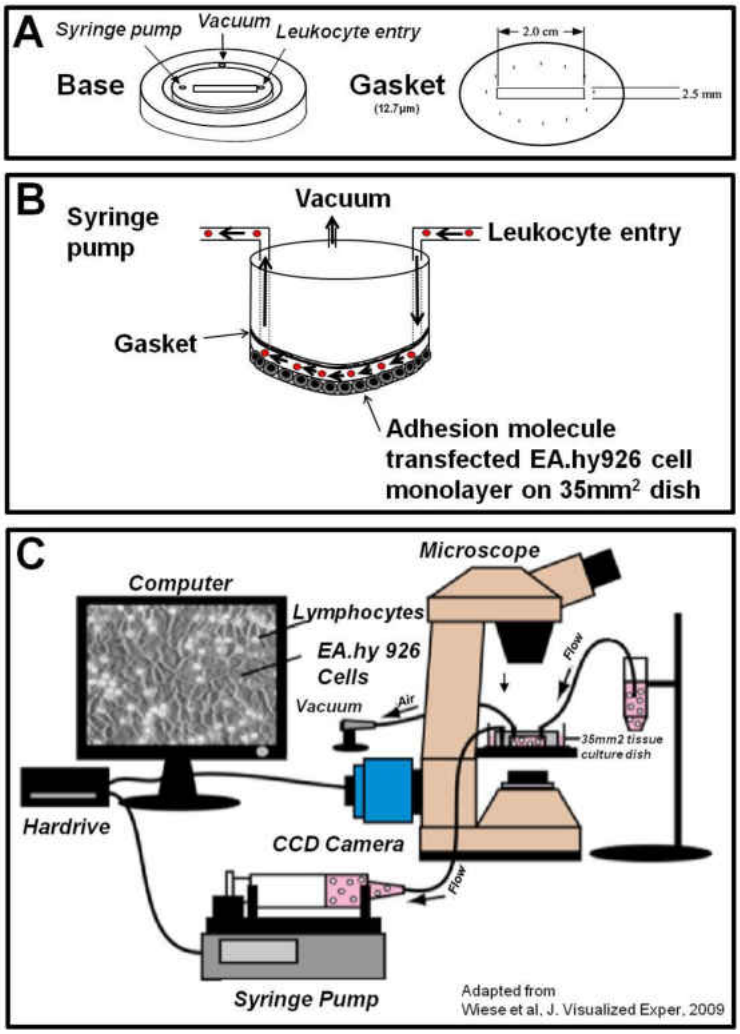


Figure 3. *In vitro* flow chamber assembly.

A) Acrylic base of flow chamber includes lymphocyte entry and exit ports, and port for vacuum seal. The base was assembled on a rubber gasket (12.7µm thick) with a viewing area of 2.0 cm x 2.5 mm. B) 926 monolayers were grown to confluence on 35 mm^2 dishes. Lymphocytes were withdrawn in laminar flow over 926 monolayers by a programmable syringe pump at defined shear stresses. C) Interactions of lymphocyte-endothelial interactions were observed on an inverted microscope, recorded using a CCD camera, and analyzed by Metaview™ software.

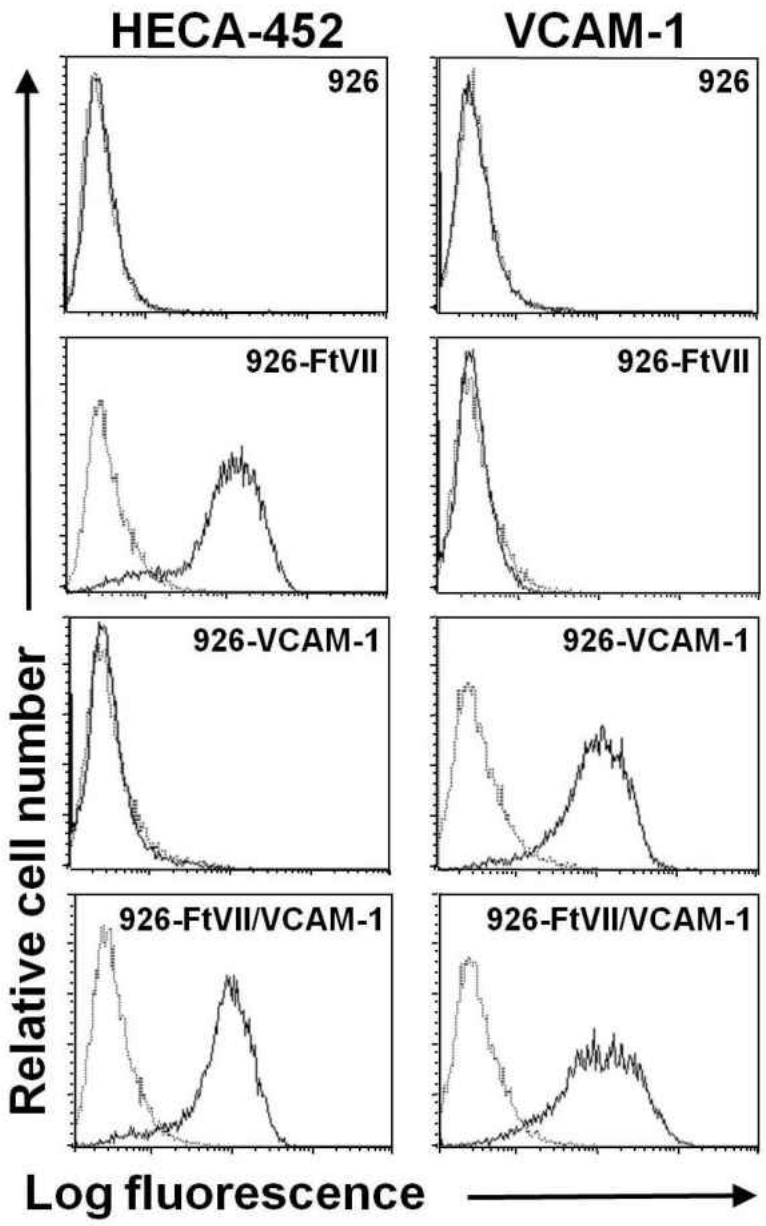


Figure 4. Adhesion molecule expression on 926 and TK-1 cell lines.

HECA-452 Ag and VCAM-1 expression on 926 cells transfected with FtVII and/or VCAM-1 cDNA. 926 cells were stained with the HECA-452 and VCAM-1 mAbs (solid lines) and direct and indirect immunofluorescence was assessed by flow cytometry. Dashed lines indicate cell staining with isotype-matched control antibodies.

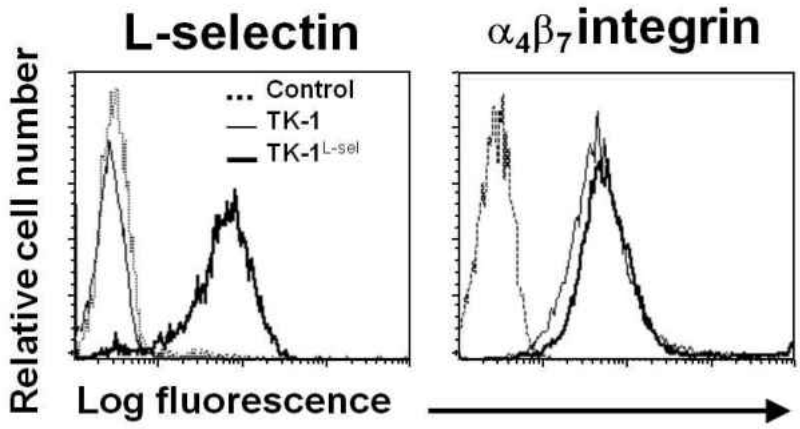


Figure 5. Adhesion molecule expression on TK-1 cell lines. TK-1 cell lines with or without transfection with human L-selectin cDNA were stained with LAM1-116 (L-selectin) and DATK32 ($\alpha_4\beta_7$ integrin) mAbs. Thin line represents TK-1 cells, thick lines represent cell TK-$1^{L\text{-}sel}$, and dashed line represents isotype-matched control mAbs. Results represent a minimum of three independent experiments.

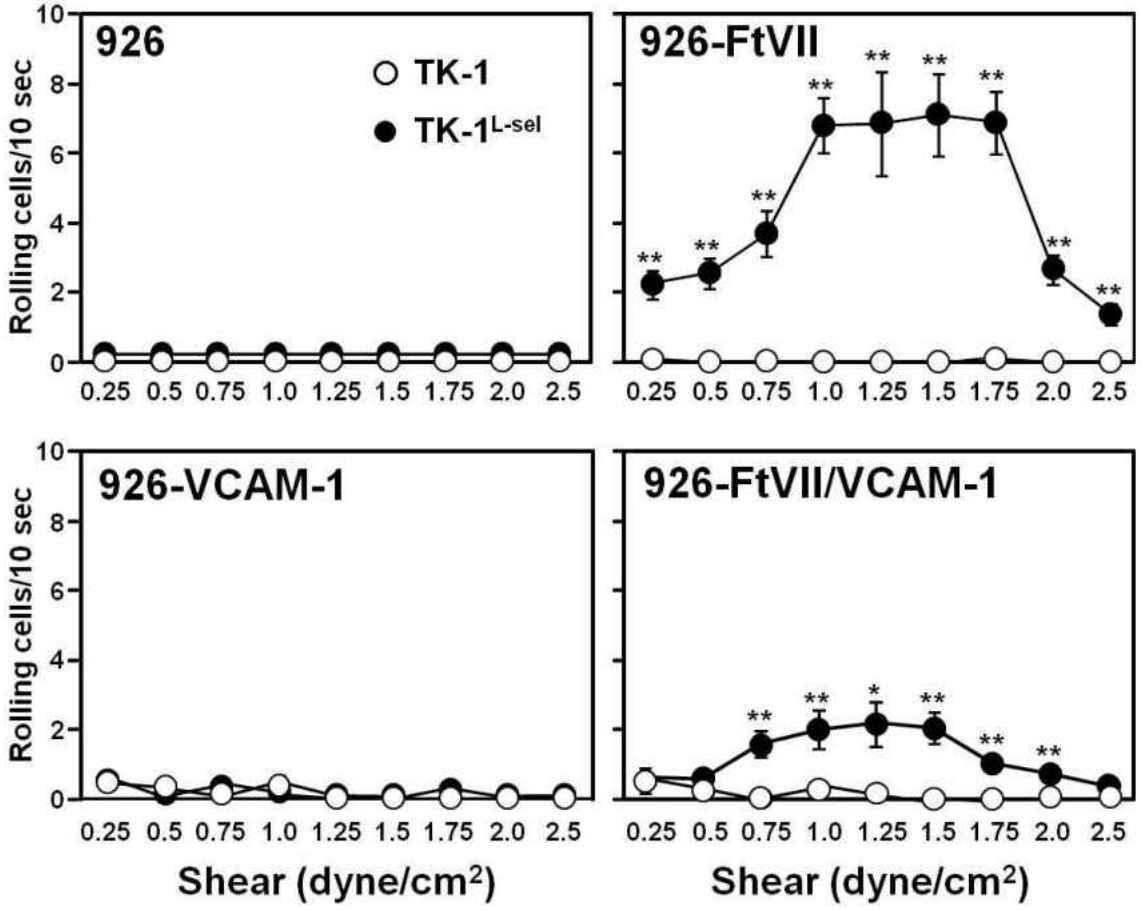


Figure 6. L-selectin expression enhances $\alpha_4\beta_7$ integrin interactions with VCAM-1. Numbers of TK-1 and TK-$1^{L\text{-}sel}$ cells rolling across the indicated monolayers of 926 cell lines under defined shear stresses. The number of rolling cells that crossed a 665-µm-wide field over a 10 s period in 3 randomly chosen fields was determined for each shear stress. Values represent the mean ± SEM results from 3-8 independent experiments. *Differences between results from all other 926 cell lines were significant; $p<0.05$. **$p<0.01$.

Figure 7. $\alpha_4\beta_7$ integrin/VCAM-1 interaction decreases L-selectin-dependent rolling velocities at low and high shear stress.

A) Representative images of lymphocyte rolling across endothelial monolayers over time. Lymphocyte velocities were determined using a digital time stamp. B) TK-$1^{L\text{-}sel}$ cell rolling velocity on EA.hy926-FtVII, -VCAM-1 or –FtVII/VCAM-1 monolayers at shear stress of 1.75 (*left*) or 0.25 dynes/cm^2 (*right*). Symbols represent the velocities of 100 random individual cells from 5 independent experiments ranked by rolling velocity. The number of TK-$1^{L\text{-}sel}$ cells that rolled on 926-VCAM-1 monolayers at 1.75 dyne/cm^2 was <100 from all experiments (data not shown). C) Mean rolling velocities of TK-$1^{L\text{-}sel}$ cells on 926 cell monolayers at the indicated shear stress. Values represent the mean ± SEM results from 3-8 independent experiments. *Differences between results were significant, $p<0.05$; **$p<0.01$.

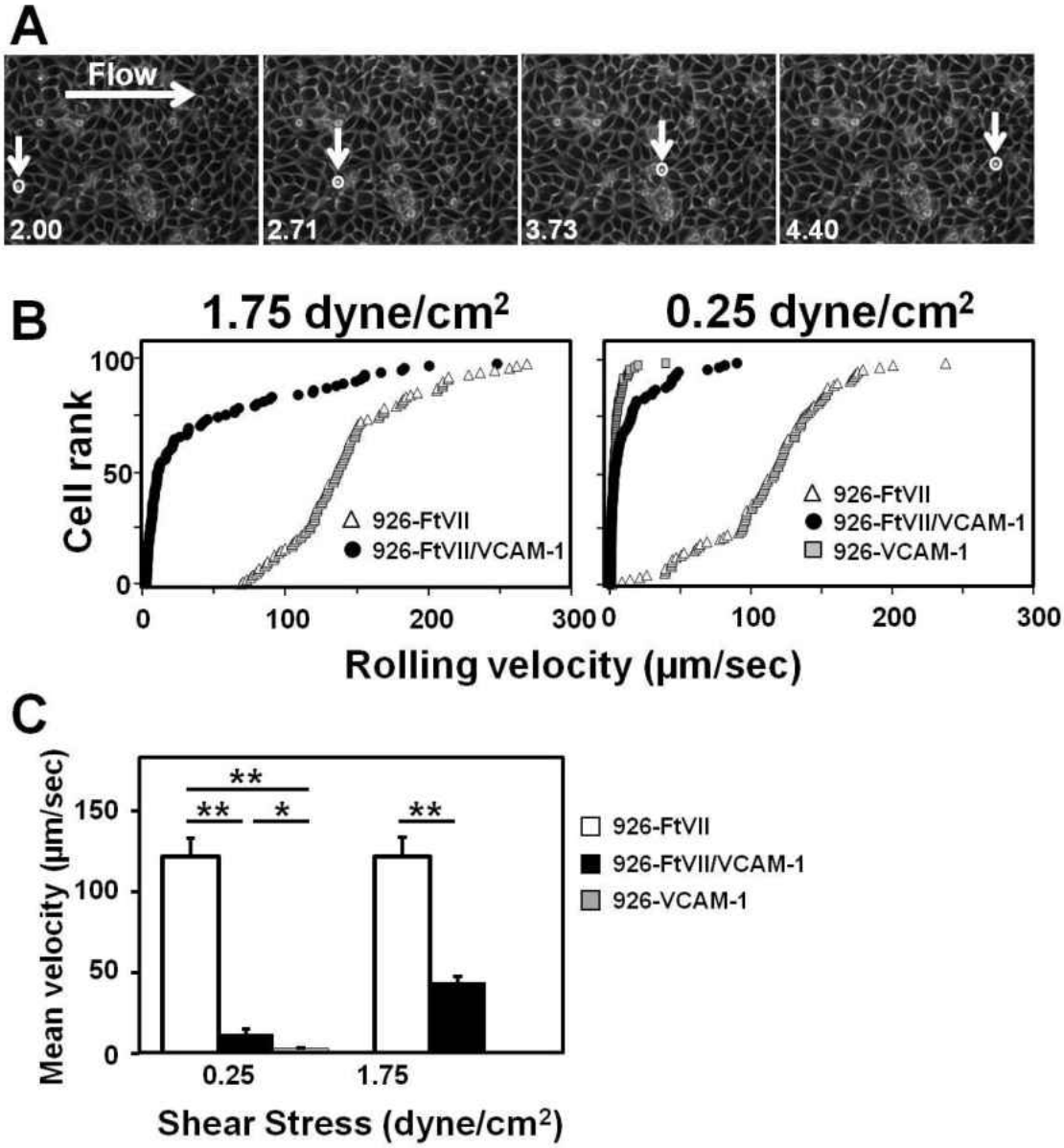

A
Flow
2.00
2.71
3.73
4.40
B
1.75 dyne/cm²
0.25 dyne/cm²
Cell rank
926-FtVII
926-FtVII/VCAM-1
926-VCAM-1
Rolling velocity (μm/sec)
C
Mean velocity (μm/sec)
**
**
*
**
926-FtVII
926-FtVII/VCAM-1
926-VCAM-1
0.25
1.75
Shear Stress (dyne/cm²)

Figure 7

Figure 8. Combined function of L-selectin and $\alpha_4\beta_7$ integrin enhances TK-1$^{L\text{-}sel}$ cell adherence at physiologic shear stress. The number of native TK-1 and L-selectin-transfected TK-1$^{L\text{-}sel}$ cells that adhered to 926 cell monolayers (circles and arrows) after a 10-min period was determined at the indicated shear stresses. A) Representative light micrographs showing TK-1 and TK-1$^{L\text{-}sel}$ cell binding to 926-VCAM-1 and B) 926-FtVII/VCAM-1 cell monolayers. Bar = 10 μm. C) Values represent the mean ± SEM results from 3-6 independent experiments. **Differences between results for TK-1 and TK-1$^{L\text{-}sel}$ cells were significant, $p<0.01$.

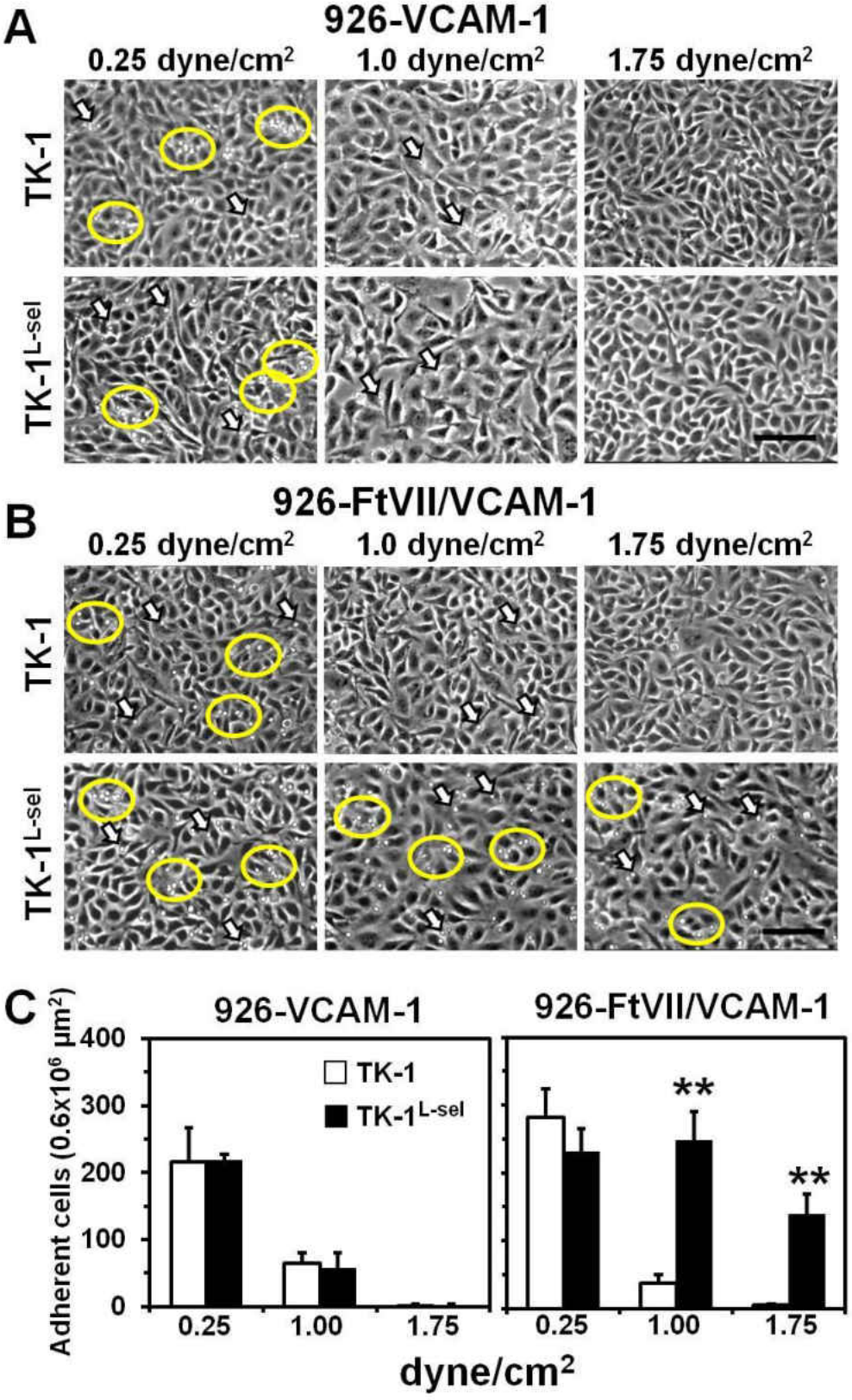

A
926-VCAM-1
0.25 dyne/cm2
1.0 dyne/cm2
1.75 dyne/cm2
TK-1
TK-1L-sel
B
926-FtVII/VCAM-1
0.25 dyne/cm2
1.0 dyne/cm2
1.75 dyne/cm2
TK-1
TK-1L-sel
C
926-VCAM-1
926-FtVII/VCAM-1
Adherent cells (0.6x10^6 µm^2)
400
300
200
100
0
TK-1
TK-1L-sel
**
**
0.25
1.00
1.75
0.25
1.00
1.75
dyne/cm2

Figure 8

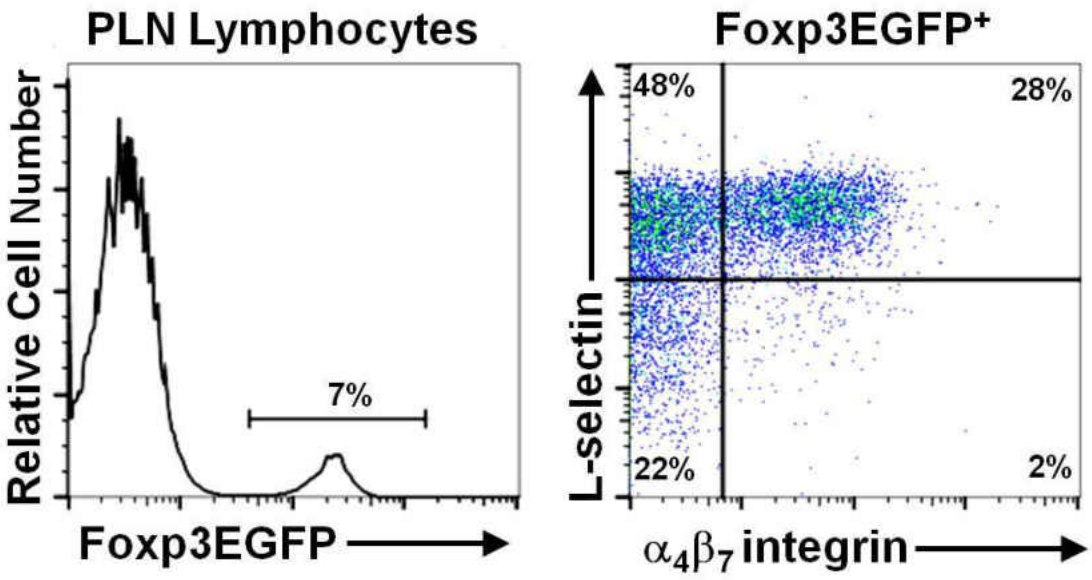


Figure 9. L-selectin and $\alpha_4\beta_7$ integrin are expressed by subsets of regulatory T cells. Murine peripheral lymph node (PLN) cells were isolated from Foxp3^{EGFP} mice and labeled with anti-L-selectin, anti-α_4 integrin, anti-β_1 integrin and anti-$\alpha_4\beta_7$ integrin mAbs. Foxp3^{EGFP+} cells indicative of Treg cell phenotype were gated (*left*) and analyzed for L-selectin and $\alpha_4\beta_7$ integrin expression (*right*). Results are representative of at least three independent experiments. Numbers indicate the frequency of cells located in each delimited quadrant.

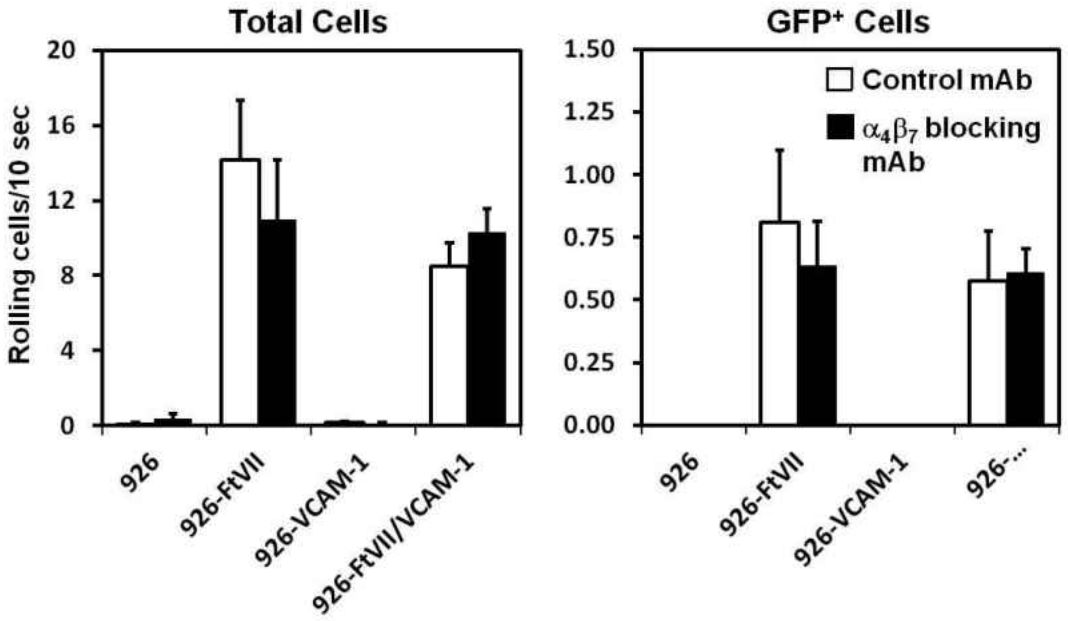


Figure 10. L-selectin dependent Treg cell rolling on EA.hy926 monolayers at physiologic shear stress.

Lymphocytes isolated from Foxp3^{EGFP} PLN were treated with 20 μg/ml $\alpha_4\beta_7$ integrin blocking or rat IgG control mAb for 15 min at 37°C. Lymphocytes were withdrawn over native 926 and 926-transfected monolayers at shear stress of 1.75 dyne/cm^2. Lymphocyte rolling was determined by counting the number of cells that crossed a 665 μm wide field over a 10 s time period. A) Total lymphocyte-endothelial monolayer interactions were assessed using phase contrast microscopy and B) Treg cell interactions were assessed by fluorescence microscopy. For each experiment, a minimum of three videos ranging from 15-45 s in random fields were analyzed and averaged. Values represent the mean ± SEM of results from 3-8 independent experiments.

Figure 11. $\alpha_4\beta_7$ integrin contributes to Treg cell adhesion to VCAM-1 at physiologic shear flow in the presence of L-selectin function.

Lymphocytes isolated from $Foxp3^{EGFP}$ PLN were treated with 20 μg/ml $\alpha_4\beta_7$ integrin blocking or rat IgG control mAb for 15 min at 37°C. Lymphocytes were withdrawn over endothelial monolayers for 10 min at a shear stress of 1.75 dyne/cm^2. A) Representative phase contrast (top) and fluorescence (bottom) light micrographs indicating $Foxp3^{EGFP-}$ lymphocyte (arrowheads) and $Foxp3^{EGFP+}$ Treg cell (arrows) adherence to 926-FtVII/VCAM-1 monolayers. Scale bar = 100 μm. B) Mean number of adherent $Foxp3^{EGFP-}$ and $Foxp3^{EGFP+}$ cells on the indicated 926 monolayers. Values represent the mean ± SEM of results from 3-8 independent experiments. *Differences between results for control mAb and $\alpha_4\beta_7$ integrin blocking mAb treated cells were significant, $p<0.05$.

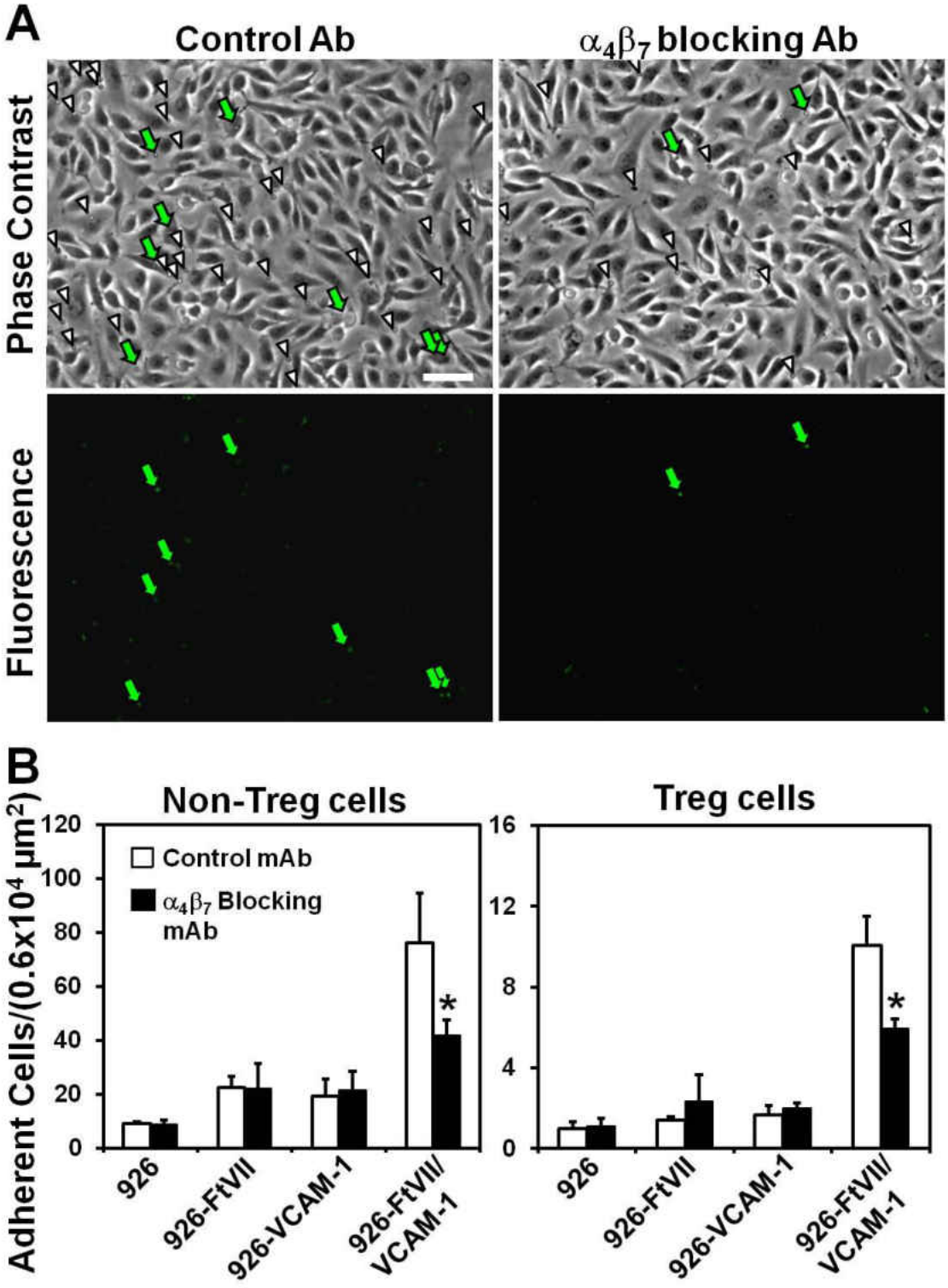

A
Control Ab
α4β7 blocking Ab
Phase Contrast
Fluorescence
B
Non-Treg cells
Treg cells
Control mAb
α4β7 Blocking mAb
Adherent Cells/(0.6x10^4 µm^2)
120
100
80
60
40
20
0
16
12
8
4
0
926
926-FtVII
926-VCAM-1
926-FtVII/ VCAM-1
*

Figure 11

CHAPTER 3

REGULATORY T CELL ACCUMULATION IN TUMORS AND SECONDARY LYMPHOID TISSUE DURING PROGRESSION OF MURINE 4T1 BREAST CANCER

ABSTRACT

Regulatory T (Treg) cells mediate tumor immune evasion by suppressing anti-tumor effector T cell responses in peripheral lymphoid tissues and within the tumor. While elevated Treg cell numbers have been shown to correlate with increased tumor growth, mechanisms that regulation their distribution within secondary lymphoid tissue and tumor tissue are not well understood. L-selectin, an adhesion molecule constitutively expressed on all classes of leukocytes, functions early in the adhesion cascade and regulates the migration of lymphocytes to lymph nodes through high endothelial venules. In addition, L-selectin can mediate migration of lymphocytes to sites of inflammation by binding to ligands present on inflamed endothelium. Treg cells express L-selectin and require L-selectin for entry into resting lymph nodes. However, the role of L-selectin in regulating Treg cell distribution into lymph nodes during chronic inflammation, such as cancer has not been examined. Therefore, we investigated the role of L-selectin in regulating the distribution patterns of Treg cells using a murine 4T1 breast cancer model at different stages of tumor progression. To assess the contribution of L-selectin in Treg cell distribution during tumor progression, forkhead box p3 (Foxp3)^{EGFP} reporter mice were crossed with L-selectin-deficient (L-selectin$^{-/-}$) mice to generate Foxp3^{EGFP}/L-selectin$^{-/-}$ mice. Mice were inoculated with 4T1 tumors and allowed to progress for 1, 2, 3, or 4 weeks. Treg cell distribution in the blood, secondary lymphoid tissues, and tumors from Foxp3^{EGFP} and Foxp3^{EGFP}/L-selectin$^{-/-}$ mice at each time point were analyzed by flow cytometry. The frequency and number of Treg cells increased in the blood and tumor-draining lymph nodes (dLN) in mice with 1, 2, 3, and 4 week tumors compared to tumor-free controls. Treg cell populations also increased with tumor tissue in mice with 2, 3,

and 4 week tumors. Importantly, Treg cell populations preferentially accumulated in dLNs compared to contra-lateral non-tumor-draining lymph nodes (ndLNs). Furthermore, $Foxp3^{EGFP}$/L-$selectin^{-/-}$ mice had significantly reduced numbers of Treg cell populations within the dLN, ndLN and within the tumor itself. Interestingly, Treg cell populations increased at higher rates than conventional $CD4^+$ T cell populations within lymph nodes and the blood as tumors progressed. These results demonstrate that L-selectin is required for normal Treg cell distribution within secondary lymphoid tissue and tumor tissue during 4T1 tumor progression. Therefore, L-selectin plays an important role in regulating Treg cell distribution during tumor progression, and may contribute to increases in Treg cells in tumors and tumor draining lymph nodes, thus promoting an immune suppressive environment.

INTRODUCTION

Regulatory T (Treg) cells play an essential role in immune homeostasis through the maintenance of immunological self-tolerance by suppressing pathological and physiological immune responses. Deficiencies in Treg cells result in a vast array of autoimmune diseases while the presence of Treg cells inhibit immune response to cancers and graft rejection (4, 360-362). Treg cells can be identified by the functionally controlled transcription factor, forkhead box P3 (Foxp3), although they have heterogeneous phenotype, function, and generation (5, 6, 8, 363). The functional importance of Foxp3 in Treg cells has been demonstrated by the onset of autoimmune diseases, graft rejection, and reduced tumor growth in Foxp3-deficient mice (11-13).

Since the 1980s, evidence for the role of Treg cells in tumor immune tolerance has been demonstrated in several models of cancer (324-326). Evasion of immune responses by tumors is mediated in part by Treg cells through inhibition of effector cells via cell-cell contact and cytokine-mediated suppression (43, 319). The importance of Treg cells in tumor progression has been demonstrated by studies in mice in which depletion of total $CD4^+$ T cells induced tumor rejection and by Treg cell antibody-depletion resulting in antitumor effects (330). Studies have clearly demonstrated elevated proportions of $CD4^+$ Treg cells in the population of tumor infiltrating leukocytes of tumor masses and tumor-draining lymph nodes (dLNs), in addition to permanent upregulation of Treg cells in patients with cancer even after cancer eradication (189, 251-253, 255, 258). Thus, the abundance of Treg cells results in immunosuppression of tumor-specific effector responses and cancer immunotherapy (265-267, 364).

Adhesion molecule expression has been characterized for subsets of Treg cells and shows that the predominant population of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells express high levels of L-selectin (192, 197, 198). The importance of L-selectin in lymphocyte migration and recirculation has been well established for conventional T cells (reviewed in 83), while its role in Treg cell distribution, recirculation, and migration has been less well defined. Specifically, it is unclear whether L-selectin mediates Treg cell distribution during conditions of chronic inflammation, such as cancer. Therefore, we sought to elucidate the role of L-selectin in regulating Treg cell distribution using the murine 4T1 mammary carcinoma model (365). The 4T1 tumor model is a widely used metastatic model that mimics stage IV human breast cancer; when injected into a Balb/c mice, it is highly aggressive and readily and spontaneously metastasizes to lung, lymph nodes, liver, bone, and other sites (365-367). Several studies have shown that Treg cells are elevated in patients with breast cancer and is correlated with poor prognosis (368, 369), and also play a role in the metastasis and development of murine 4T1 carcinomas, thereby decreasing subsequent long-term survival (370, 371).

In this study, we determined the role of L-selectin in regulating Treg cell distribution during 4T1 tumor progression using both wild type and L-selectin-deficient (L-selectin$^{-/-}$) $Foxp3^{EGFP}$ reporter mice. Specifically, Treg cell number and frequency were determined in the spleen, blood, tumor tissue, and dLN and non-tumor draining lymph nodes (ndLN) during 1, 2, 3 and 4 weeks of 4T1 tumor progression. Results showed that the number of Treg cells increased during progressive stages of tumor growth in both wild type and L-selectin$^{-/-}$ mice. Treg cells preferentially accumulated in dLN in all stages of tumor progression. Furthermore, L-selectin loss resulted in severe

deficiencies in Treg cell distribution within the dLN and ndLN, as well as tumor tissue and was accompanied by a compensatory increase in Treg cell numbers in the spleen. These results not only confirm a preferential accumulation of Treg cells within dLNs, but also that L-selectin is required for optimal Treg cell population distribution within these lymph nodes during stages of tumor progression. Furthermore, the presence of Treg cell populations within the tumor tissue is, at least in part, L-selectin dependent. These results demonstrate preferential Treg cell accumulation within dLNs, that may result in an increased immunosuppressive environment.

MATERIALS AND METHODS

Animals

Balb/c and Balb/cFoxp3^{EGFP} (Foxp3^{EGFP}) mice (346) were purchased from The Jackson Laboratory (Bar Harbor, ME). The L-selectin null mutation (129) from a C57BL/6 strain background was backcrossed more than 10 generations onto the Balb/c strain background. Balb/c/L-selectin$^{-/-}$ mice were then intercrossed with Balb/cFoxp3^{EGFP} mice to produce Foxp3$^{EGFP+/+}$L-selectin$^{-/-}$ (Foxp3^{EGFP}/L-selectin$^{-/-}$) homozygous mice. The Foxp3^{EGFP} genotype was assessed by PCR analysis of genomic DNA from ear biopsies and lymphocyte cell-surface expression of L-selectin was assessed by staining of blood leukocytes with the fluorescein isothiocyanate (FITC)-conjugated LAM1-116 mAb (125) and analyzed by flow cytometry. For all studies, female mice 8-12 weeks of age were used. All mice were maintained under specific pathogen-free conditions and screened regularly for pathogens. All studies and procedures were approved by the Animal Care and Use Committee of the University of Wisconsin-Milwaukee.

Murine 4T1 cell line and tumor induction

The 4T1 murine tumor cell line was purchased from ATCC (CRL-2539, Manassas, VA) and used to induce solid tumors in Balb/c, Foxp3^{EGFP}, and Foxp3^{EGFP}/L-selectin$^{-/-}$ female mice. 4T1 cells were maintained in complete RPMI medium (RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml streptomycin, all from Invitrogen, Gaithersburg, MD; and 2-mercaptoethanol, Sigma-Aldrich, St. Louis, MO). Cells were grown in a humidified incubator supplied with 5% CO_2 at 37ºC to 70% confluence. 4T1 cells were non-enzymatically lifted using cellstripper™ (Invitrogen) and resuspended ($0.2x10^6$/ml) in

supplement-free RPMI medium for injections. Adult female mice (8-12 week old) were injected subcutaneously with $1x10^4$ 4T1 cells in 50 µl in the lower left mammary fat pad. Tumors were measured by caliper, and tumor volume (mm^3) were calculated using the following equation: $LxW^2/2$. Mice were sacrificed 1, 2, 3, or 4 weeks after tumor inoculation.

Lymphocyte isolation and flow cytometry

Control (tumor-free) and 1-, 2-, 3- and 4-week tumor-bearing mice were sacrificed and blood, spleen, mesenteric lymph nodes (MLN, superior mesenteric cords), tumor, dLN, non-tumor ndLN and Peyer's patches were harvested for analysis. Single-cell suspensions of tissues were prepared as previously described (82). Briefly, spleen and lymph nodes were minced with needles and filtered through 70 µm nylon mesh and washed twice with PBS. For splenocyte suspensions, erythrocytes were lysed with Tris-buffered 0.1M ammonium chloride solution. Peyer's patches were incubated in Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution supplemented with HEPES (15 µM), FBS (10%; all from Invitrogen) and EDTA (5 µM) for 30 min at 37ºC. Peyer's patches were mechanically dissociated with frosted glass slides, filtered through nylon mesh, and washed twice with phosphate-buffered saline (PBS). Tumors were harvested, minced with scissors, and incubated for 1 hour at 37ºC in collagenase buffer (20.5 U/µl DNAse, 562 U/ml collagenase A, both from Sigma Aldrich, and 5% (w/v) bovine serum albumin, ThermoFisher, Waltham, MA). Transfer pipettes were used to shear-dissociate tumor tissues prior to filtering through nylon mesh, and washing twice with PBS. Cell counts for all tissues were determined using a hemocytometer. For blood samples, leukocytes were enumerated after lysis in 2% acetic acid. Single-cell suspensions of all tissues were

stained with PE-conjugated anti-mouse CD4 (RM4-5, BD Biosciences, Mountain View, CA). Isotype-matched rat IgG antibodies were used as controls for non-specific staining (Southern Biotech, Birmingham, AL). Cells with forward and side light scattered properties of mononuclear cells were gated and the total number and frequency of $CD4^+EGFP^-$ and $CD4^+EGFP^+$ cells from each tissue was determined using a FACSCalibur flow cytometer using CellQuest™ Pro software (BD Biosciences, Mountain view, CA) and analysis was performed using FlowJo™ analysis software (Tree Star Inc., Ashland, OR). Cell numbers for lymphocyte populations were obtained by multiplying the frequency of the cell populations by the total cell count of the tissue.

Statistical analysis

All data are presented as mean values ± SEM. Significant differences between sample means were determined using one-way ANOVA and the Student's-*t* test. Comparisons were made between control and each experimental time point. A paired Student's *t*-test was used for comparisons between results of ndLN and dLN within the same animal. A p value of <0.05 was considered to be statistically significant.

RESULTS

$CD4^+$ T cells and Treg cells increase in the spleen during progression of 4T1 tumors

Immune responses to tumors can be seen through changes in specific populations of lymphocytes within peripheral and mucosal lymphoid tissues, at sites of inflammation and within the blood. While increases in $CD4^+$ T cells indicate anti-tumor immune responses, accumulation of Treg cells oppose and suppress these responses. Therefore, to characterize changes in both $CD4^+$ T cells and Treg cell populations during tumor progression, $Foxp3^{EGFP}$ mice were inoculated with 4T1 tumors and tissues were harvested at 1, 2, 3 or 4-weeks following tumor induction. Immune responses to the tumor resulted in changes in overall tissue cellularity during tumor progression (Table I). In general, tumor progression had little to no affect on cellularity of mucosal lymphoid tissues such as MLN and Peyer's patches of wild type or L-$selectin^{-/-}$ mice (data not shown); therefore, no further analyses were performed.

Within the spleen, $CD4^+$ T cell frequency significantly decreased (by 15%), by week 2 and further decreased (by 20%) by weeks 3-4 after 4T1 inoculation (Fig. 12A *left*). Despite the significant decrease in frequency of $CD4^+$ T cells during tumor progression, the total number of $CD4^+$ T cells steadily and significantly increased in mice bearing 1 through 3-week tumors after tumor inoculation by 1.2-, 1.5-, and ~2-fold, respectively, compared to control (Fig. 12A *right*). By week 4 post-4T1 inoculation, the number of $CD4^+$ T cells had sharply increased (by 4.5-fold) compared to control mice and all other tumor-bearing mice ($p<0.01$). The decrease in frequency but increase in total number of $CD4^+$ T cells can be explained by the large increase in total spleen cellularity that occurred during tumor progression (Table I). The finding that the

frequency of $CD4^+$ T cells decreased during tumor progression indicates that other cell populations were expanding at a greater rate or that new populations (such as myeloid-derived suppressor cells) were being generated.

In the same manner, the frequency of Treg cells in the spleen declined during tumor progression reaching a maximal decrease of 12% compared to control by week 4 (Fig. 12B *left*). While the number of Treg cells in 1-week tumor-bearing mice did not change, the number of Treg cells increased significantly in 2- through 4-week tumor-bearing mice compared to control mice (Fig. 12B *right*). Specifically, numbers of splenic Treg cells increased by 1.6- and 2.2-fold in mice bearing 2- and 3-week tumors, compared to control mice, respectively. In mice bearing 4-week-old tumors, Treg cell numbers were dramatically increased compared to control mice (4.8-fold) and significantly increased compared to all other stage tumor mice ($p<0.01$). Interestingly, despite these large changes in cell numbers, the ratio of Treg to conventional $CD4^+$ T cells remained constant (Fig. 12C). These results indicate that both $CD4^+$ T cells and Treg cell populations undergo dramatic but proportional increases in the spleen resulting in a maintenance of the balance between Treg and conventional $CD4^+$ T cells during 4T1 tumor progression.

CD4 T and Treg cells preferentially increase in tumor-draining lymph nodes

Initial and local immune responses during inflammation occur within the adjacent tissue-draining lymph nodes. During tumor progression, dLNs progressively increased in total cellularity, while ndLNs had little to no change (Table I). To directly compare $CD4^+$

T cell and Treg cell distribution in lymph nodes during tumor progression, cell numbers were compared between left inguinal dLNs and right contra-lateral ndLNs.

Within the dLN, the frequency of $CD4^+$ T cells showed a similar trend as that observed in the spleen with significant decreases in frequency occurring over time with a maximum of a 26% decline in mice with 4-week tumors compared to control mice (data not shown). By contrast, the contra-lateral ndLN showed no difference in $CD4^+$ T cell frequency during tumor progression. During tumor progression, the number of $CD4^+$ T cells in the ndLNs remained constant during the first 3 weeks of tumor progression, with a modest decline 4 weeks after tumor induction. In contrast to the ndLN, $CD4^+$ T cell numbers significantly increased in dLNs by 2.2-fold after 2 weeks, and peaked at 2.8-fold in 3-week tumor-bearing mice (Fig. 13A *left*). This preferential increase in $CD4^+$ T cell numbers within dLNs compared to ndLNs resulted in a 2-fold difference in 2-week tumor-bearing mice, and this difference was further amplified during tumor progression, with a 2.8-fold difference observed in 3-week tumor-bearing mice and a 3.6-fold difference in mice with 4-week tumors (Fig. 13A *left*).

In contrast to $CD4^+$ T cells, Treg cell frequency showed only modest changes during tumor progression in both ndLNs and dLNs in mice with tumors progressed up to 3 weeks (data not shown). In the ndLN, this resulted in the total number of Treg cells remaining similar to control mice (Fig. 13A *right*); importantly, the number of Treg cells within the dLNs steadily increased with tumor progression, plateauing at 3.8-fold increase over control mice by week 3 and 4 following tumor inoculation (Fig. 13A *right*).

Similar to what was observed for $CD4^+$ T cells, Treg cell numbers were preferentially increased in dLNs compared to ndLNs during tumor progression. Specifically, Treg cell numbers were 2.3-fold higher in dLN compared to contra-lateral ndLNs in 2-week tumor-bearing mice, 3-fold higher in mice bearing 3-week tumors, and 4.4-fold higher with 4-week tumors (Fig. 13A *right*). Importantly, while the numbers of $CD4^+$ T cells and Treg cells both increased during tumor progression, Treg cell numbers increased to a greater extent than $CD4^+$ T cells in 3- and 4-week tumor mice. This disproportionate increase in Treg cells resulted in an increased ratio of Treg to conventional $CD4^+$ T cells within the dLNs in mice at these time points (by 1.3-fold and 1.7 -fold, respectively, Fig. 13B). Thus, while both $CD4^+$ T cells and Treg cells specifically accumulate in dLNs, the greater accumulation may result in a more suppressive environment within dLNs.

Circulating $CD4^+$ T cells and Treg cells increase during 4T1 tumor progression

To determine the circulating number and frequency of $CD4^+$ T cells and Treg cells, blood samples were collected from mice with progressive stages of 4T1 tumors. $CD4^+$ T cell frequency was increased in 1-week tumor-bearing mice, but returned to control levels thereafter (Fig. 14A *left*). While the frequency of circulating $CD4^+$ T cells during the later stages of tumor progression remained relatively constant, the number of these cells increased markedly during tumor progression, with increases of 2.6-fold in 3-week tumor-bearing mice and 4.5-fold in 4-week tumor-bearing mice (Fig. 14A *right*). This result is reflective of the drastic increase in overall number of circulating cells in the mice at these time points (Table I).

By contrast to $CD4^+$ T cells, Treg cell frequency increased significantly with tumor progression (Fig. 14B *left*). Specifically, compared to tumor-free mice, Treg cell frequency in the blood increased by ~1.2-fold in 1-3-week tumor-bearing mice, with a further increase to 1.7-fold in mice with 4-week tumors. Concurrent with an increase in Treg cell frequency, the number of circulating Treg cells increased at each stage of tumor progression (Fig. 14B *right*). Mice bearing 1- and 2-week tumors mice showed similar increases in numbers of Treg cells (1.5- and 1.6-fold, respectively), while the number of Tregs in mice bearing 3- and 4-week tumors was increased by 3.3- and 7.5-fold, respectively. While both $CD4^+$ T cells and Treg cells increased in the blood during tumor progression, Treg cell numbers increased to a greater extent than $CD4^+$ T cells in mice bearing 3- and 4-week tumors resulting in a significant increase in the Treg cell to $CD4^+$ T cell ratio (Fig. 14C). Thus, greater increases in circulating Treg cell populations compared to $CD4^+$ T cells suggest a systemic skewing favoring suppressive cells during tumor progression.

Numbers of $CD4^+$ T cells and Treg cells increase within 4T1 tumor tissue

As tumors increase in size, blood and lymphatic vessels also increase to support the growing tumor. Tumor infiltrating leukocytes function to clear malignant cells and promote anti-tumor immune responses. However, immune responses to tumors can change drastically within the tumor microenvironment. Therefore, changes in $CD4^+$ T cell and Treg cell populations were analyzed within 4T1 tumors over time. Solid tumors were harvested, digested in collagenase buffer, and cell populations were quantified by flow cytometry. On average, tumor volume increased rapidly during tumor progression with volumes of 15 mm^3 at 1 week, and increased to 54 mm^3, 286 mm^3, and 787 mm^3 by

2, 3 and 4 weeks, respectively. As expected, the number of total infiltrating leukocytes within the tumor increased significantly with time: by 7.5-fold from 1 to 2 weeks, 2.4-fold from 2 to 3 weeks, and another 2.9-fold from 3 to 4 weeks (Table 1). The number of $CD4^+$ T cells within tumor tissue did not change between 1- and 2-week tumors, but increased by 3.6-fold in 3-week tumors and 8.1-fold in 4-week tumors compared to 1-week tumors (Fig. 15A *left*). Similarly, the number of Treg cells within the tumor tissue steadily increased during tumor progression, with the greatest rise in 3-week tumors (by 7.3-fold) and 4-week tumors (by 17.2-fold) compared to 1-week tumors (Fig 15A *right*). Due to the lack of $CD4^+$ T cell number increase between 1- and 2-week tumors, but steady rise in Treg cell numbers, the Treg/$CD4^+$ T cell ratio in mice with 2- through 4-week tumors were significantly greater than 1-week tumors (Fig 15B). In fact, the Treg;$CD4^+$ T cell ratio within the tumor tissue was higher than that observed in any other tissue or blood, suggesting that there is a greater suppressive environment within solid tumor tissues.

$CD4^+$ T cells and Treg cells increase within the spleen in L-selectin$^{-/-}$ mice during stages of 4T1 tumor progression

L-selectin is required for almost all lymphocyte entry into resting lymph nodes, and is important in lymphocyte migration to peripheral tissue where L-selectin ligands are expressed. However, the role of L-selectin in lymphocyte distribution in secondary lymphoid tissue, blood, and solid tumor tissue during 4T1 tumor progression has not been defined. Therefore, to determine the role of L-selectin in both frequency and number of $CD4^+$ T cell and Treg cells during tumor progression, Foxp3^{EGFP} mice and Foxp3^{EGFP}/L-selectin$^{-/-}$ mice with advancing stages of tumor progression were used.

Within the spleen, $CD4^+$ T cell frequency decreased during tumor progression, with significant decreases occurring with mice bearing 1, 2, 3 and 4-week tumors (reduced by 21%, 22%, 42%, and 53% respectively, Fig. 16A *left*). By contrast, the number of $CD4^+$ T cells only increased significantly (by 70%) in mice bearing 4-week tumors (Fig. 16A *right*). While overall spleen cellularity increased dramatically during tumor progression (Table 1), the decline in frequency resulted in little change in numbers of $CD4^+$ T cells until 4 weeks after tumor inoculation.

Treg cell frequency in the spleen of $Foxp3^{EGFP}$/L-$selectin^{-/-}$ mice did not change at any week following 4T1 inoculation (Fig. 16B *left*). However, the number of Treg cells slowly rose during the first 3 weeks of tumor progression, with a significant increase in mice bearing 2- and 3-week tumors (by 1.4-fold and 1.8-fold, respectively); and spiked significantly higher in mice bearing 4-week tumors (by 3.1-fold) compared to tumor-free mice (Fig. 16B *right*). The relatively modest change in $CD4^+$ T cell and Treg cell numbers during the first 3 weeks of tumor progression did not significantly affect the Treg to $CD4^+$ T cell ratio of the spleen (Fig. 16C). However, in mice bearing 4-week tumors, the Treg to $CD4^+$ T cell ratio was significantly increased (by 1.9-fold) compared to control mice. Therefore, in L-$selectin^{-/-}$ mice, the splenic environment may become increasingly immunosuppressive during late stages of tumor progression.

$CD4^+$ T cells and Treg cell populations increase within the tumor-draining lymph nodes in L-$selectin^{-/-}$ mice during 4T1 tumor progression

To determine the role of L-selectin in regulating $CD4^+$ T cell and Treg cell accumulation in lymph nodes during tumor progression, left inguinal dLNs, and contra-

lateral right inguinal ndLNs from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice were compared at different stages of tumor growth. In general, the cellularity of lymph nodes from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice was drastically reduced compared to Foxp3^{EGFP} mice (Table I). Despite low cell numbers, the cellularity of the dLN increased during tumor progression, while the ndLN only showed a modest increase in 3- and 4-week tumor-bearing mice. Within both the ndLNs and dLNs of Foxp3^{EGFP}/L-selectin$^{-/-}$ mice, the frequency of CD4^{+} T cells remained relatively constant throughout tumor progression (data not shown). However, when comparing CD4^{+} T cell accumulation between ndLNs and dLNs, CD4^{+} T cell numbers were preferentially increased (by 2.8- and 4.8-fold) in dLNs in Foxp3^{EGFP}/L-selectin$^{-/-}$ mice with 3 and 4-week tumors, respectively (Fig. 17A *left*).

In contrast to CD4^{+} T cells, Treg cell frequency increased during tumor progression in both ndLNs and dLNs in Foxp3^{EGFP}/L-selectin$^{-/-}$ mice (data not shown). In the ndLNs, the total number of Treg cells showed modest increases as the tumor progressed (Fig. 17A *right*). By contrast, increases in Treg cells within dLNs were markedly higher with a 19-fold increase occurring in 3- and 4-week tumor-bearing mice compared to tumor-free mice ($p<0.01$). This preferential increase in dLNs resulted in a significant increase in Treg cell accumulation in mice bearing 3-week tumors (by 4.6-fold) and 4-week tumors (by 5.2-fold).

Importantly, Treg cells increased in dLNs to a greater extent than did CD4^{+} T cells in 3 and 4-week tumor mice. This disproportionate increase in Treg cells resulted in an increased ratio of Treg to conventional CD4+ T cell within the dLNs in mice bearing 3- and 4-week tumors by 2.4-fold and 2.2-fold, respectively. (Fig. 17B). These results suggest, that while L-selectin deficiency results in severely reduced cellularity of lymph

nodes, $CD4^+$ T cell and Treg cells still preferentially accumulate in dLNs compared to ndLNs. Furthermore, Treg cells increase to a greater extent within dLNs during late stages of tumor progression, promoting an immune suppressive environment.

Circulating $CD4^+$ T cells and Treg cells increase within tumor-bearing Foxp3EGFP/L-selectin$^{-/-}$ mice

To determine whether L-selectin deficiency influenced circulating $CD4^+$ T cell or Treg cell populations, blood samples were collected from mice with different stages of tumor progression. The frequency of $CD4^+$ T cells in Foxp3^{EGFP}/L-selectin$^{-/-}$ mice transiently increased in 1-week tumor-bearing mice (by 56%), but returned to near control levels by week 2 (Fig. 18A *left*). While the frequency remained constant during most stages of tumor progression, the number of circulating $CD4^+$ T cells was significantly increased in early stage tumors (1 week by 2.1-fold and 2 week by 2.6-fold), and peaked in mice bearing 3-week tumors (9.4-fold, Fig. 18A *right*). This increase in circulating $CD4^+$ T cells is consistent with a dramatic increase in total circulating leukocytes within the blood of tumor-bearing mice (Table I).

By contrast, circulating Treg cell frequencies of Foxp3^{EGFP}/L-selectin$^{-/-}$ mice steadily increased during tumor progression with significant increases occurring in mice bearing 3 and 4-week tumors (Fig. 18B *left*). Similarly, the number of circulating Treg cells within the blood of Foxp3^{EGFP}/L-selectin$^{-/-}$ mice increased significantly with tumor progression starting at week 2 and reaching increase of ~14-fold by weeks 3 and 4 post-tumor inoculation (Fig. 18B *right*). The relatively larger increases in numbers of Treg cells compared to $CD4^+$ T cells resulted in significantly increased ratio of Treg to $CD4^+$ T

cells in 3 and 4-week tumor-bearing Foxp3^{EGFP}/L-selectin$^{-/-}$ mice (Fig. 18C). Thus, greater increases in circulating Treg cell populations compared to CD4^{+} T cells suggests that systemically, suppressive cells are favored during tumor progression in Foxp3^{EGFP}/L-selectin$^{-/-}$ mice.

CD4^{+} T cells and Treg cell populations increase within 4T1 tumors of Foxp3^{EGFP}/L-selectin$^{-/-}$ mice during 4T1 tumor progression

To assess the contribution of L-selectin to CD4^{+} T cell and Treg cell infiltration into tumors, tumors from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice were harvested at progressive stages of tumor development. In 1-week tumors, very few CD4^{+} T cells were recovered from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice (Fig. 19A *left*). However, in 2-week tumors, the number of CD4^{+} T cells significantly increased (by 95-fold) compared to 1-week tumors. Interestingly, the number of CD4^{+} T cells was highest in 2-week tumors despite a large increase in tumor cellularity in mice with 3- and 4-week tumors (Table 1).

Similar to CD4^{+} T cells, very few Treg cells were recovered from early 1-week tumors, but significantly more Treg cells accumulated in 2- and 3-week tumors (Fig. 19A *right*). Compared to 1-week tumors, Treg cell numbers were increased over 100-fold in 2- through 4-week tumors. Interestingly, the Treg/CD4^{+} T cell ratio did not significantly change in the tumor tissue during tumor progression (Fig. 19B).

L-selectin deficiency alters lymphocyte populations in the spleen

To determine the effect of L-selectin on CD4^{+} T cell and Treg cell accumulation in the spleen during tumor progression, Treg and CD4+ T cell numbers within the spleen in wild type (Fig. 12) and L-selectin$^{-/-}$ mice (Fig. 16) were directly compared. Compared

to wild type Foxp3^{EGFP} mice, CD4^{+} T cell numbers were markedly increased in tumor-free Foxp3^{EGFP}/L-selectin$^{-/-}$ mice, as previously described for L-selectin deficiency on the C57BL/6 background (82). This increase was maintained in mice bearing 1-, 2- and 3-week tumors (Fig. 20 *left*). Interestingly, 4 weeks after tumor inoculation, there was no significant difference in numbers of CD4^{+} T cells between Foxp3^{EGFP} mice and Foxp3^{EGFP}/L-selectin$^{-/-}$ mice due to a large increase in wild type animals that did not occur in the L-selectin$^{-/-}$ mice.

Similar to CD4^{+} T cell numbers, spleen Treg cell numbers were modestly higher in Foxp3^{EGFP}/L-selectin$^{-/-}$ mice compared Foxp3^{EGFP} mice and this increase remained constant during the first 3 weeks after tumor inoculation, and then was lost (Fig 20 *right*). Therefore, L-selectin deficiency resulted in an increased number of splenic CD4^{+} T cells and Treg cells, suggesting that these cells are unable to migrate into peripheral tissues.

L-selectin is required for optimal CD4^{+} T cell and Treg cell accumulation tumor-draining lymph nodes

To determine the magnitude of the L-selectin-dependent lymphocyte response in the lymph nodes of tumor-bearing mice, the accumulation of CD4^{+} T cells and Treg cells into the dLNs of Foxp3^{EGFP} mice (Fig. 13) and Foxp3^{EGFP}/L-selectin$^{-/-}$ mice (Fig. 17) were directly compared. As expected, the number of CD4^{+} T cells in the lymph nodes was significantly reduced in Foxp3^{EGFP}/L-selectin$^{-/-}$ mice compared to Foxp3^{EGFP} mice in tumor-free and tumor-bearing mice (Fig. 21 *left*). Importantly, the number of CD4^{+} T cells remained dramatically reduced in the dLNs of Foxp3^{EGFP}/L-selectin$^{-/-}$ mice compared to Foxp3^{EGFP} mice at all times during tumor progression. Similar to CD4^{+} T

cell numbers, the Treg cell numbers in dLNs were drastically reduced in the absence of L-selectin in both tumor-free and tumor-bearing mice during all stages of tumor progression (Fig. 21 *right*). Interestingly, $CD4^+$ T cell and Treg cell numbers increased relatively more in $Foxp3^{EGFP}$/L-$selectin^{-/-}$ mice compared to $Foxp3^{EGFP}$ mice during tumor progression, although this may be a result of the extremely low numbers of starting cells in the L-$selectin^{-/-}$ animals. These results indicate that L-selectin is required for the vast majority of $CD4^+$ T cell and Treg cell entry into dLNs during tumor progression. However, this requirement is somewhat reduced in late stage tumor-bearing mice, suggesting other factors are contributing to cell accumulation within the dLNs.

L-selectin deficiency contributes to $CD4^+$ T cell and Treg cell accumulation in late stage 4T1 tumors

To determine the role of L-selectin in $CD4^+$ T cells and Treg cell accumulation within solid 4T1 tumors, $CD4^+$ T cell and Treg cell numbers from $Foxp3^{EGFP}$ (Fig. 15) and Foxp3EGPF/L-$selectin^{-/-}$ mice (Fig. 19) were directly compared. In $Foxp3^{EGFP}$ mice, $CD4^+$ T cell and Treg cell numbers with the tumor increased significantly during tumor progression. By contrast, $CD4^+$ T cell and Treg cell numbers in $Foxp3^{EGPF}$/L-$selectin^{-/-}$ mice increased only between 1 and 2 weeks, but did not further increase with tumor progression. This difference in cell accumulation resulted in a 60-68% reduction ($p=0.1$) of both $CD4^+$ T cell and Treg cell tumor-resident cells in $Foxp3^{EGFP}$/L-$selectin^{-/-}$ mice bearing 3-week tumors compared to $Foxp3^{EGFP}$ mice., and a significant reduction (by 85-88%) in $Foxp3^{EGFP}$/L-$selectin^{-/-}$ mice bearing 4-week tumors. Thus, L-selectin appears to play little if any role in lymphocyte accumulation within early-stage tumors, but plays an

important role in accumulation of both $CD4^+$ T cell and Treg cell into solid tumor tissue in late-stage tumors.

DISCUSSION

Treg cells suppress immune responses and are important for maintaining immunologic homeostasis. However in cancer, the presence and accumulation of suppressive Treg cells allows tumors to evade immune responses thereby promoting tumor growth and progression. L-selectin is an adhesion molecule that functions early in the adhesion cascade and is required for lymphocyte migration through HEV in lymph nodes and migration to sites of inflammation. Treg cells express high levels of L-selectin (Chapter 2, Ref. 192, 197), but whether this expression influences their distribution in secondary lymphoid and tumor tissue during tumor progression is not known. Therefore, we sought to determine the role of L-selectin in Treg cell accumulation in secondary lymphoid tissue, and tumor tissue during progression of a murine breast cancer model.

To determine the role of L-selectin in regulating Treg cell accumulation during tumor progression, $Foxp3^{EGFP}$/L-$selectin^{-/-}$ mice were generated. Both wild type and L-$selectin^{-/-}$ mice were inoculated with 4T1 tumor cells and allowed to progress for 1, 2, 3, or 4 weeks. We demonstrate that Treg cells preferentially accumulated within dLNs compared to conventional $CD4^+$ T cell populations, suggesting that immunosuppressive environment was promoted during tumor progression (Figs. 15 and 20). Importantly, L-selectin was required for the vast majority of Treg cell accumulation within the dLNs of mice during tumor progression (Fig. 21B). In addition, L-selectin significantly contributed to Treg cell accumulation within solid tumor tissue at late stages of progression (Fig. 22).

However, $CD4^+$ T cell and Treg cell populations were significantly increased within the spleens of tumor-free and tumor-bearing L-selectin$^{-/-}$ mice compared to wild type mice. This is consistent with previous studies that have demonstrated increased cellularity within the spleen of L-selectin$^{-/-}$ mice, due to the inability of these cells to migrate into peripheral lymphoid tissue (82). The increase in numbers of splenic Treg cells is in conjunction with a severe decrease in Treg cell distribution within peripheral ndLNs and dLNs and indicates that L-selectin functions in normal Treg cell distribution to peripheral lymphoid tissue. However, in the absence of L-selectin, Treg cell populations increased at a faster rate than the $CD4^+$ T cells within the spleen (Fig. 16), suggesting that Treg cells may be more dependent on L-selectin function for accumulation within peripheral lymphoid tissue than conventional $CD4^+$ T cells. Surprisingly, in mice bearing 4-week tumors, numbers of both $CD4^+$ T cells and Treg cells in the spleen were equivalent between wild type and L-selectin$^{-/-}$ mice. This finding is most likely explained by an increase in Treg cell generation within wild type mice rather than a redistribution of Treg cells since no corresponding large decreases were observed in other tissues.

L-selectin deficiency resulted in drastically reduced $CD4^+$ T cell and Treg cell accumulation within ndLNs and dLNs. Interestingly, there was a greater rate of increase in Treg cell accumulation within dLNs of late stage tumor-bearing L-selectin$^{-/-}$ mice compared to wild type mice, resulting in smaller differences between these genotypes late during tumor progression. This could be attributed to a greater degree of Treg cell generation within dLNs of L-selectin$^{-/-}$ mice, reduction in apoptosis, or L-selectin-independent migration. L-selectin-independent migration of Treg cells into lymph nodes

may occur through HEV, or may be due to afferent lymphatic drainage from the tumor tissue.

During tumor progression, local immune responses in the nearby lymph nodes results in an increase in lymph node cellularity due, in part, to activation and expansion of cells in response to tumor antigens (232). Furthermore, studies have demonstrated both $CD4^+$ T cells and Treg cells are primed in dLN during tumor progression (189). We sought to determine whether these changes in $CD4^+$ T cell and Treg cells populations were specific to dLNs compared to contra-lateral ndLNs, and if L-selectin affected cellular distribution to these tissues. It is important to note that cellularity in the dLN increased in both wild type and L-selectin$^{-/-}$ mice throughout tumor progression, while the ndLNs showed only a modest increase during early tumor progression. In wild type mice and L-selectin$^{-/-}$ mice, both $CD4^+$ T cells and Treg cells accumulated preferentially in dLNs compared to ndLNs, and this accumulation increased as tumors progressed. Taken together, these data suggest a role for both L-selectin-dependent and -independent mechanisms in regulation of $CD4^+$ T cell and Treg cell preferential accumulation to dLN during tumor progression.

However, Treg cell accumulation differed from conventional $CD4^+$ T cell accumulation during tumor progression for both wild type and L-selectin$^{-/-}$ mice. Within the blood, ndLNs and dLNs, Treg cell number and frequency increased during tumor progression in both wild type and L-selectin$^{-/-}$ mice while $CD4^+$ T cell frequencies remained the same or declined, indicating that the Treg cell population increased at a faster rate than $CD4^+$ T cells. Therefore, the increase in Treg cell populations was not due to overall increased cellularity, but rather was specific to Treg cell populations.

Both anti-tumor and suppressor cells enter into tumor tissue and play a role in tumor growth and progression (261, 262, 266). In early stage tumors, very few $CD4^+$ T cell or Treg cell populations were detected. However, within the tumor tissue by week 2, both populations increased dramatically, along with tissue cellularity as the tumors progressed. Interestingly, $CD4^+$ T cell populations declined after 2 weeks, while Treg cells remained the same. The decline in $CD4^+$ T cell numbers resulted in Treg cell numbers being present at a higher proportion than $CD4^+$ T cells during later stages of tumor progression, supporting previous studies that have demonstrated that accumulation of Treg cells at tumor sites and dLN suppress anti-tumor immune responses (259, 272). We next determined the role of L-selectin in $CD4^+$ T cell and Treg cell population accumulation within solid tumor tissue. Interestingly, L-selectin was required for Treg accumulation within solid tumor tissue, as demonstrated by a decrease in tumor-infiltrating Treg and $CD4^+$ T cells in tumors from L-selectin$^{-/-}$ mice compared to the number of cells found in tumors from wild type mice.

The origin of Treg cells within tumor sites and within local lymphoid tissue remains an area of debate. Some studies suggest that induced Treg (iTreg) cell populations may differentiate from naïve $CD4^+$ T cells after interaction with natural Treg (nTreg) cells or by polarization induced by the cytokine milieu of the tumor environment (14, 268-270). The complex tumor microenvironment functions to reprogram many types of cells, including naïve and effector T cells, macrophages, and B cells which creates an environment that suppresses immune responses to tumors (274, 275, 281-283). Tumors also host a large number of cells, including tumor-infiltrating leukocytes, tumor-associated macrophages, natural killer cells, and other inflammatory cells that infiltrate

the tumors and produce cytokines and modulate pro- or anti-tumorigenic responses (276-280). However, it is unclear if induction, rather than specific recruitment of Treg cells to tumors, results in their accumulation within tumor tissue and dLNs.

Alternatively, specific Treg cell recruitment to tumors and tumor-associated lymphoid tissue may account for an increase number of Treg cells found within these tissues. In fact, specific recruitment of Treg cells to sites of tumors and surrounding lymphoid tissue fosters a suppressive environment for effector responses (266, 327-329). Indeed, selective stimuli such as chemokines, adhesion molecules, and receptors mediate the migration of Treg cells to secondary lymphoid tissue and tumor tissue (192, 372, 373). Hence, mechanisms regulating Treg cell migration into tumor and dLNs may be the greatest contributing factor in Treg cell accumulation at these sites.

Table I. [a]To determine the role of L-selectin on Treg cell distribution within tumor and secondary lymphoid tissue, $Foxp3^{EGFP}$ (Wild type) and $Foxp3^{EGFP}$/L-$selectin^{-/-}$ (L-$selectin^{-/-}$) mice were inoculated with 4T1 tumor cells in lower left mammary fat pad and tumors were allowed to progress for 1, 2, 3, or 4 weeks. For tumor-free mice (control) and each time point, tissues were harvested and total tissue cellularity was determined. *$p<0.05$, **$p<0.01$ vs. wild type. Data represents the mean ± SEM from 3-22 independent experiments.

[b]Abbreviations used: dLN, tumor-draining lymph node, ndLN, non-tumor draining lymph node, MLN, mesenteric lymph nodes, PP, Peyer's patches.

[c]Cell number x 10^6

Table I. Cellularity in Foxp3^{EGFP} and Foxp3^{EGFP}/L-selectin$^{-/-}$ mice during 4T1 tumor progression[a]

Spleen					
L-selectin Genotype	**Control[c]**	**1 week**	**2 week**	**3 week**	**4 week**
Wild type	63.3 ± 3.5	84.4 ± 4.8	132.6 ± 15.3	249.4 ± 34.6	763.0 ± 109.1
L-selectin$^{-/-}$	132.3 ± 17.3**	130.5 ± 38.7**	222.9± 40.9*	420.5 ± 80*	1,260.4± 161.7**
dLN[b]					
L-selectin Genotype	**Control**	**1 week**	**2 week**	**3 week**	**4 week**
Wild type	3.52 ± 0.40	10.36 ± 2.36	13.49 ± 2.65	18.64 ± 1.83	15.26 ± 2.78
L-selectin$^{-/-}$	0.07± 0.01**	0.23 ± 0.16*	0.27 ± 0.1**	0.47 ± 0.06**	0.84 ± 0.24**
ndLN					
L-selectin Genotype	**Control**	**1 week**	**2 week**	**3 week**	**4 week**
Wild type	3.89 ± 0.34	5.82 ± 0.68	5.17 ± 0.87	5.00 ± 0.51	3.05 ± 0.27
L-selectin$^{-/-}$	0.03 ± 0.01**	0.05 ± 0.02**	0.11 ± 0.03**	0.15 ± 0.03**	0.15 ± 0.04**
MLN					
L-selectin Genotype	**Control**	**1 week**	**2 week**	**3 week**	**4 week**
Wild type	19.94 ± 2.51	26.60 ± 2.54	24.57 ± 3.35	25.42 ± 2.35	25.52 ± 3.61
L-selectin$^{-/-}$	5.25 ± 1.66*	27.17 ± 9.05	32.54 ± 10.76	23.28 ± 2.3	16.73 ± 2.41*
PP					
L-selectin Genotype	**Control**	**1 week**	**2 week**	**3 week**	**4 week**
Wild type	3.52 ± 0.26	3.82 ± 0.33	3.08 ± 0.32	3.82 ± 0.44	3.58 ± 0.55
L-selectin$^{-/-}$	4.96 ± 0.48*	3.96 ± 0.73	6.25 ± 0.96*	2.08 ± 0.83	4.73 ± 2.98*
Blood					
L-selectin Genotype	**Control**	**1 week**	**2 week**	**3 week**	**4 week**
Wild type	6.30 ± 0.47	7.64 ± 0.80	11.37 ± 1.71	36.79 ± 5.54	106.12 ± 20.90
L-selectin$^{-/-}$	5.03 ± 1.05	5.49 ± 2.76	9.81 ± 2.76	59.60 ± 7.18	222.17 ± 53.14
Tumor					
L-selectin Genotype		**1 week**	**2 week**	**3 week**	**4 week**
Wild type		0.58 ± 0.16	4.36 ± 1.73	10.45 ± 1.92	29.83 ± 5.84
L-selectin$^{-/-}$		0.07 ± 0.03	7.05 ± 1.71	14.53 ± 4.23	38.08 ± 16.84**

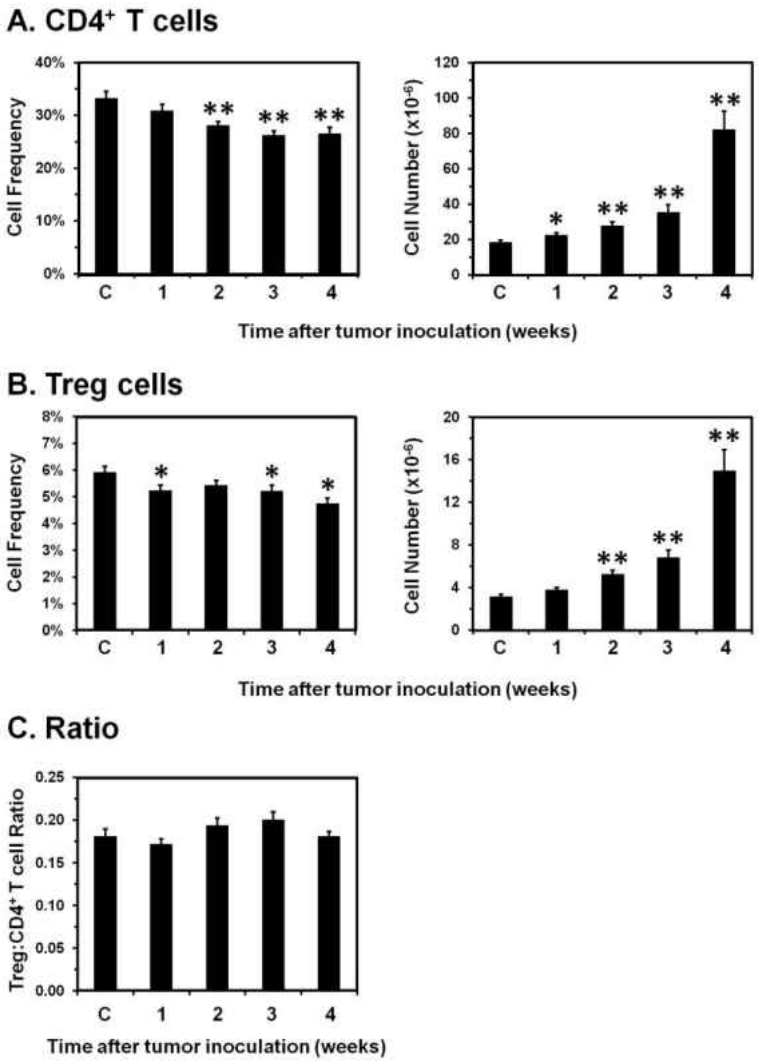


Figure 12. CD4$^+$ T cells and Treg cell numbers increase in the spleen during 4T1 tumor progression.

Spleens were harvested from Foxp3^{EGFP} mice at different stages during 4T1 tumor progression and subsets were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) CD4$^+$ T cell frequency of total lymphocytes and cell number of CD4$^+$ T cells. B) Treg cell frequency of total lymphocytes and total Treg cell number. C) Ratio of Treg/CD4$^+$ T cells. *$p<0.05$, **$p<0.01$ vs. control. Results represent means ± SEM from 3-20 independent experiments per time point.

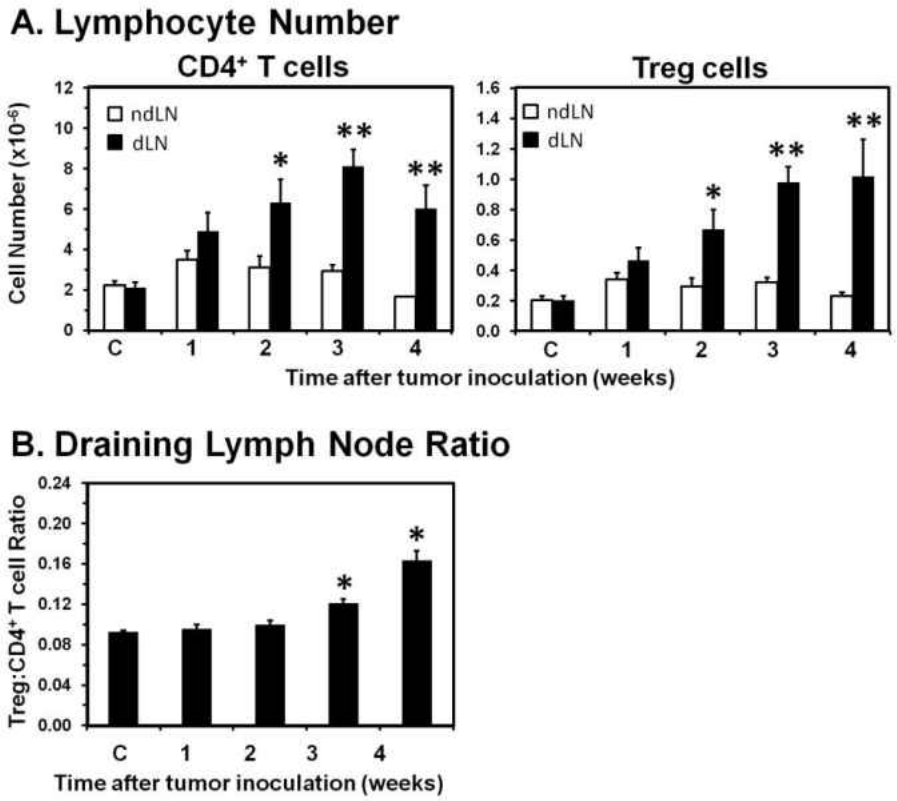


Figure 13. CD4$^+$ T and Treg cells preferentially accumulate in tumor-draining lymph nodes during 4T1 tumor progression.

Lymph nodes were harvested from Foxp3^{EGFP} mice with different stages of 4T1 tumor progression and subsets were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) Left inguinal non-tumor draining lymph nodes (ndLN) and right inguinal tumor-draining lymph nodes (dLN) were analyzed for number of CD4$^+$ T cells (left) and Treg cells (right). For comparisons between contralateral ndLN and dLNs at each time point, results were compared using a paired Student's *t*-test. *$p<0.05$, **$p<0.01$ vs. ndLN. B) Ratio of Treg/CD4$^+$ T cells. *$p<0.05$ vs. control. Results represent means ± SEM from 3-20 independent experiments per time point.

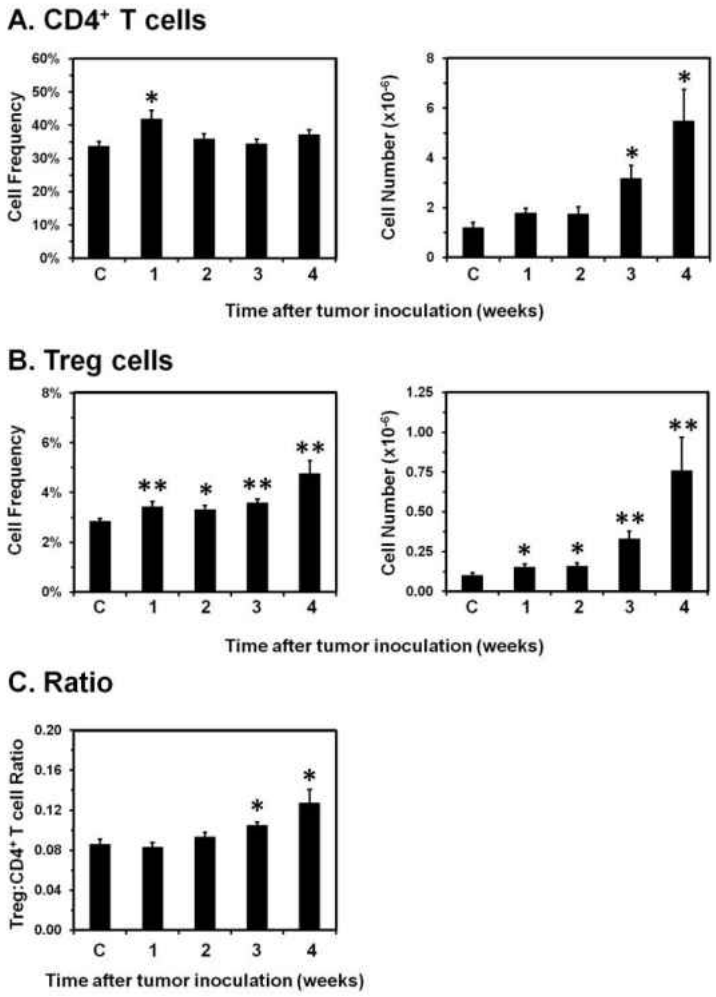


Figure 14. Circulating Treg cell populations increase at a faster rate than CD4$^+$ T cell populations in the blood during 4T1 tumor progression.

Blood samples were collected from Foxp3^{EGFP} mice with different stages of 4T1 tumor progression and subsets were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) CD4$^+$ T cell frequency of total lymphocytes and cell number of CD4$^+$ T cells. B) Treg cell frequency of total lymphocytes and total Treg cell number. C) Ratio of Treg/CD4$^+$ T cells. *$p<0.05$, **$p<0.01$ vs. control. Results represent means ± SEM from 3-20 independent experiments per time point.

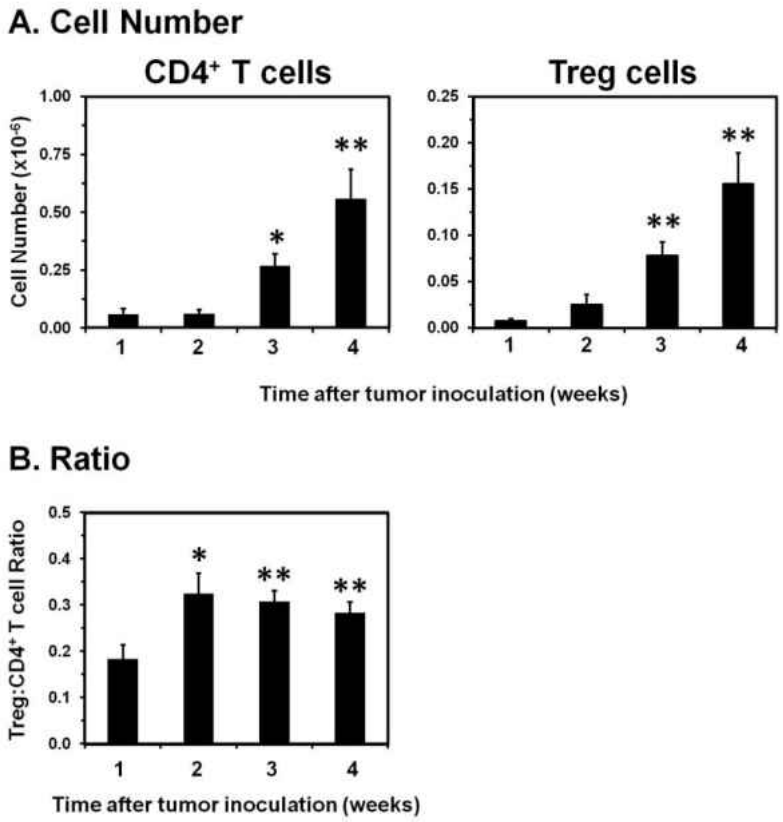


Figure 15. CD4$^+$ T cell and Treg cell populations increase in 4T1 tumor tissue during tumor progression.

4T1 tumors were harvested from Foxp3^{EGFP} mice with different stages of 4T1 tumor progression, digested in collagenase buffer, and subset cell counts and total numbers were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) CD4$^+$ T cell frequency of total lymphocytes and cell number of CD4$^+$ T cells (*left*) Treg cell frequency of total lymphocytes and total Treg cell number (*right*). B) Ratio of Treg/CD4$^+$ T cells. *$p<0.05$, **$p<0.01$ vs. control. Results represent means ± SEM from 3-20 independent experiments per time point.

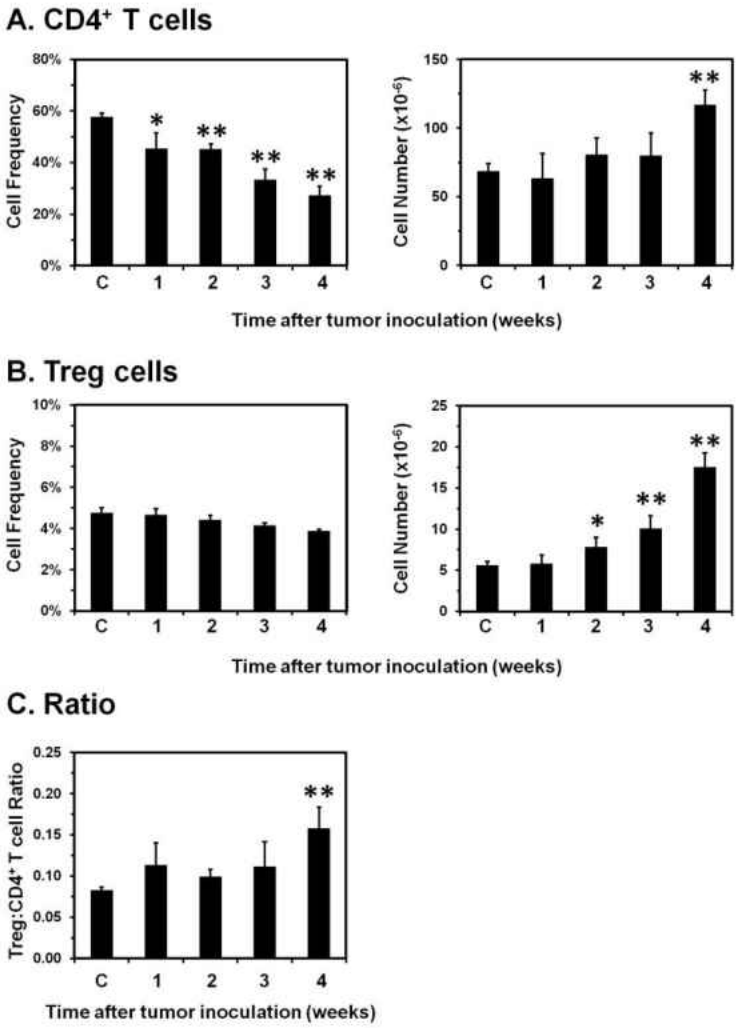


Figure 16. CD4$^+$ T cell and Treg cells increase in the spleen of L-selectin$^{-/-}$ during 4T1 tumor progression.

Spleens were harvested from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice with different stages of 4T1 tumor progression and subsets were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) CD4$^+$ T cell frequency of total lymphocytes and cell number of CD4$^+$ T cells. B) Treg cell frequency of total lymphocytes and total Treg cell number. C) Ratio of Treg/CD4$^+$ T cells. *$p<0.05$, **$p<0.01$ vs. control. Results represent means ± SEM from 3-6 independent experiments per time point.

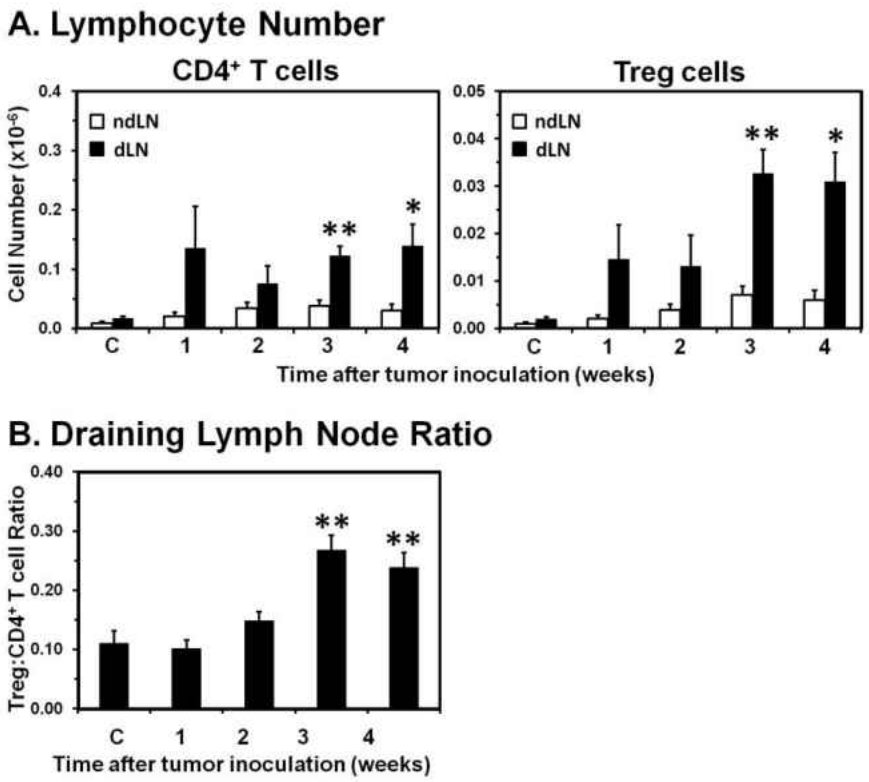


Figure 17. CD4$^+$ T and Treg cells preferentially accumulate in tumor-draining lymph nodes during 4T1 tumor progression in L-selectin$^{-/-}$ mice.

Lymph nodes were harvested from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice and subset cell numbers and frequency were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. Left inguinal non-tumor draining lymph nodes (ndLN) and right inguinal tumor-draining lymph nodes (dLN) were analyzed for number of A) CD4$^+$ T cells and (*left*) Treg cells (*right*). B) Ratio of Treg/CD4$^+$ T cells. *p<0.05, **p<0.01 vs. ndLN. Results represent means ± SEM from 3-20 independent experiments per time point.

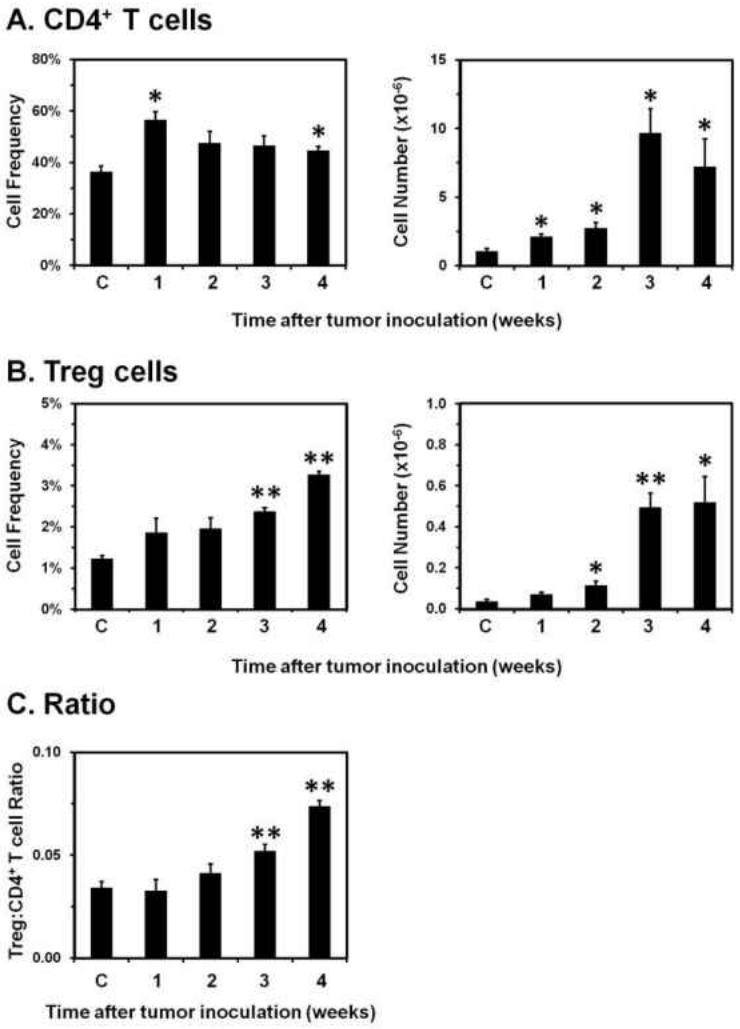


Figure 18. Circulating Treg cell populations increase at a faster rate than CD4$^+$ T cell populations in the blood during 4T1 tumor progression in L-selectin$^{-/-}$ mice.

Blood samples were collected from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice with different stages of 4T1 tumor progression and subsets were analyzed by flow cytometry. CD4$^+$ T cells and Treg cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) CD4$^+$ T cell frequency of total lymphocytes and cell number of CD4$^+$ T cells. B) Treg cell frequency of total lymphocytes and total Treg cell number. C) Ratio of Treg/CD4$^+$ T cells. *$p<0.05$, **$p<0.01$ vs. control. Results represent means ± SEM from 3-6 independent experiments per time point.

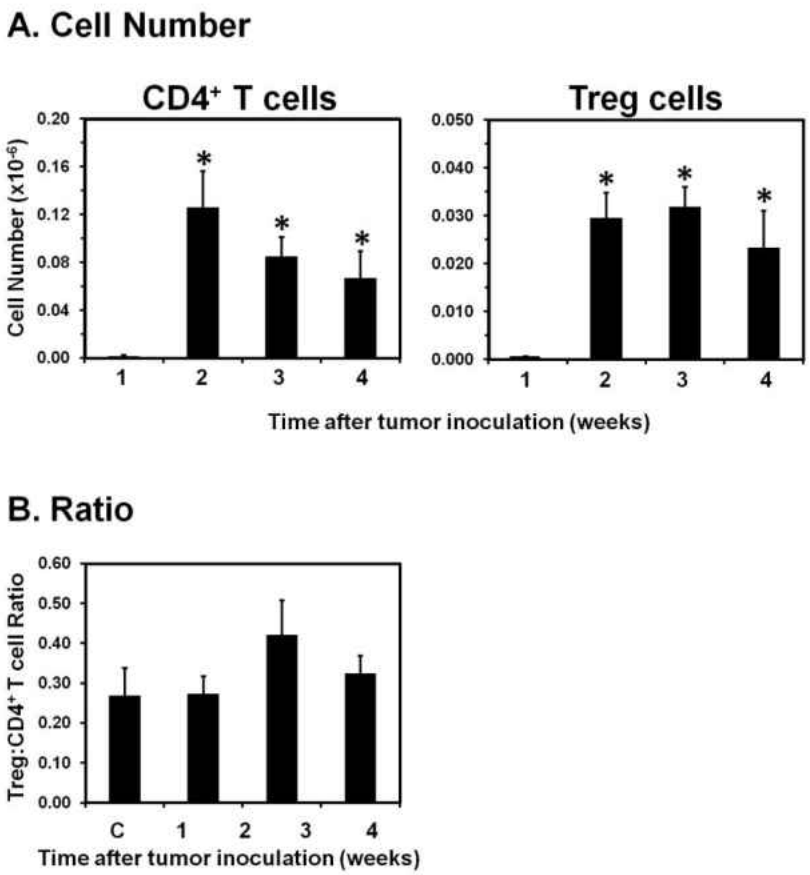


Figure 19. $CD4^+$ T cell and Treg cell populations initially in tumor tissue of L-selectin$^{-/-}$ mice with 4T1 tumors.

4T1 tumors were harvested from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice with different stages of tumor progression, digested in collagenase buffer, and subset cell numbers were analyzed by flow cytometry. Treg cells and $CD4^+$ T cells were determined using fluorochrome-conjugated antibodies to detect CD4 and by GFP expression. A) Total numbers of $CD4^+$ T cells (*left*) and Treg cells (*right*). B) Ratio of Treg/$CD4^+$ T cells. *$p<0.05$, **$p<0.01$ vs. control. Results represent means ± SEM from 3-6 independent experiments per time point.

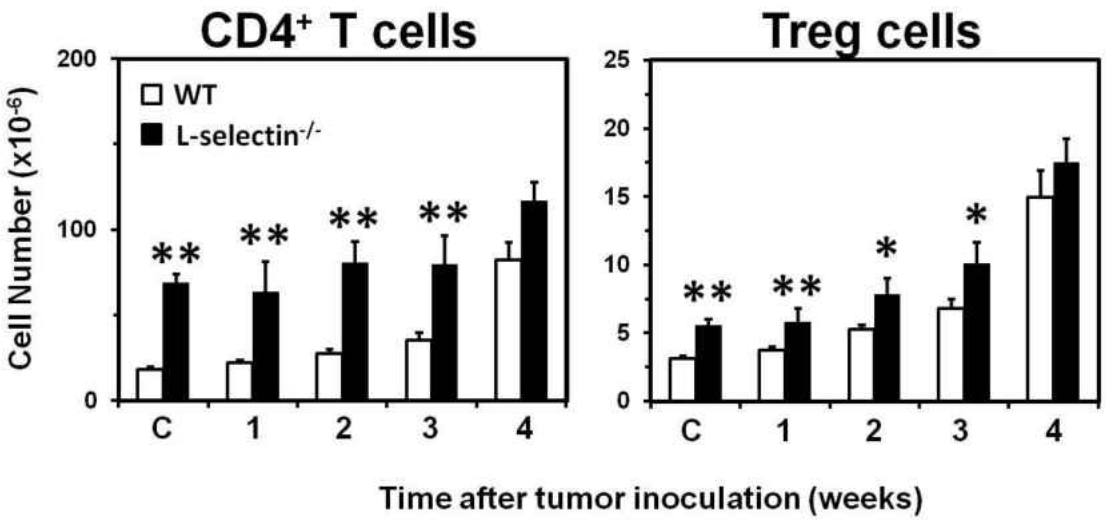


Figure 20. L-selectin deficiency increases CD4$^+$ T cell and Treg cell numbers within the spleen in mice with 4T1 tumors.

Comparison of the results shown in Figs. 12 and 16. Foxp3^{EGFP} (WT) and Foxp3^{EGFP}/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) comparison of CD4$^+$ T cells (*left*) and Treg cells (*right*). *$p<0.05$, **$p<0.01$ vs. wild type. Results represent means ± SEM from 3-20 independent experiments per time point.

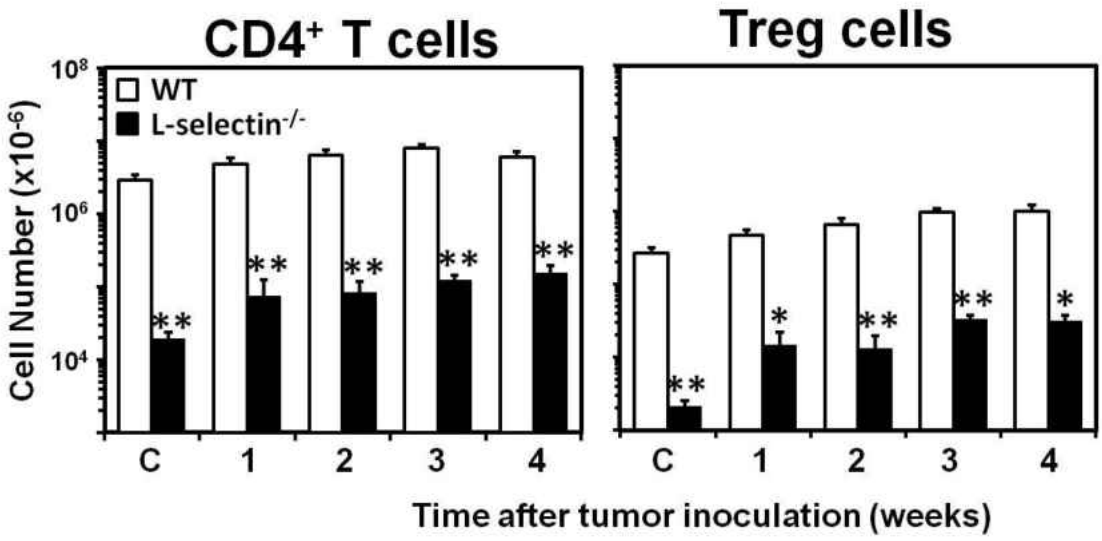


Figure 21. L-selectin is required for normal CD4$^+$ T cell and Treg cell accumulation in tumor-draining lymph nodes during 4T1 tumor progression.

Comparison of the results shown in Figs. 13 and 17 on a log scale. Foxp3^{EGFP} (WT) and Foxp3^{EGFP}/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) comparison of CD4$^+$ T cells (*left*) and Treg cells (*right*). *$p<0.05$, **$p<0.01$ vs. wild type. Results represent means ± SEM from 3-20 independent experiments per time point.

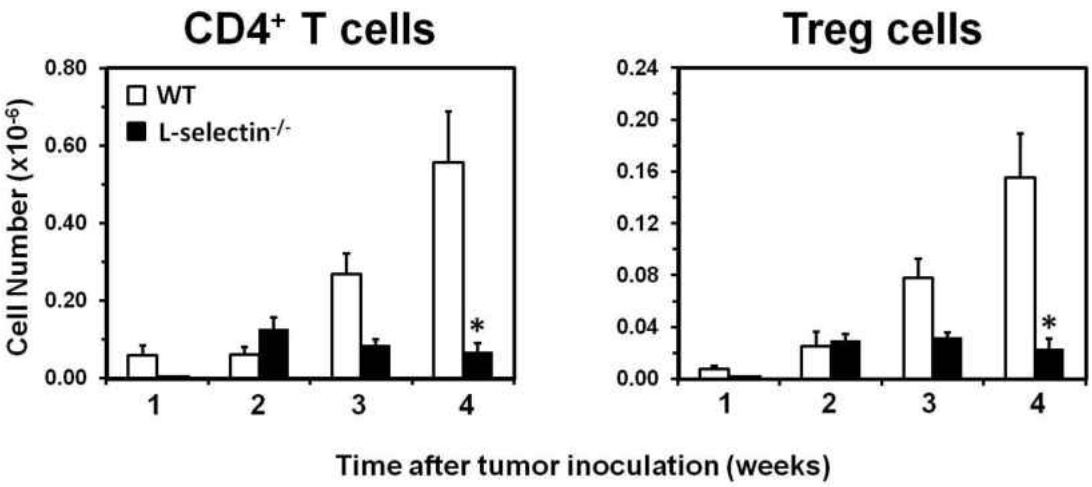


Figure 22. L-selectin is required for CD4$^+$ T cell and Treg cell accumulation in tumors during late stages of 4T1 tumor progression.

Comparison of the results shown in Figs. 15 and 19. Foxp3^{EGFP} (WT) and Foxp3^{EGFP}/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) comparison of CD4$^+$ T cells (*left*) and Treg cells (*right*). *$p<0.05$, **$p<0.01$ vs. wild type. Results represent means ± SEM from 3-20 independent experiments per time point.

CHAPTER 4

REGULATORY T CELL MIGRATION TO TUMORS AND SECONDARY LYMPHOID TISSUE DURING PROGRESSION OF MURINE 4T1 BREAST CANCER

ABSTRACT

During inflammation, robust immune responses direct the elimination of foreign pathogens and malignant cells. However, suppressor cells, such as regulatory T (Treg) cells dampen immune responses through secretion of anti-inflammatory cytokines and inhibition of effector cells via cell-cell contact mechanisms. While Treg cells are important for maintaining immunologic homeostasis, they also can inhibit immune-mediated clearance of tumor cells. Treg cell populations increase within tumor microenvironments and tumor-draining lymph nodes (dLN). However, the origin of Treg cells within these tissue is not fully understood. Treg cell-specific homing to tumors and dLNs may contribute to an increase in cell number, thereby suppressing immune responses to tumors. Lymphocyte migration to sites of inflammation and secondary lymphoid tissue is directed by expression of adhesion molecules. Specifically, L-selectin is required for lymphocyte entry into lymph nodes through binding to ligands present on high endothelial venules (HEV). In the present studies, L-selectin-dependent Treg cell migration to secondary lymphoid tissue and tumor tissues was examined. Treg cells demonstrated a propensity to migration to tumor tissue and dLNs that was amplified during late stages of tumor progression. Importantly, Treg cell migration into dLNs occurred primarily from the blood through HEV and was L-selectin-dependent. However, Treg cells were also able to enter dLNs from the tumor through afferent lymphatics. Additionally, L-selectin contributed to Treg cell migration into solid tumor tissue during late stages of tumor progression. Therefore, these studies demonstrate that one mechanism of Treg cell influx into tumor and dLNs is through specific homing of Treg cells to these tissues. Furthermore, this migration is dependent on Treg cell expression of

L-selectin. These studies provide further insight into the mechanisms of Treg cell migration to tumor tissue and dLNs may lead to a better understanding of tumor immune evasion and provide new targets for immunotherapeutic strategies.

INTRODUCTION

Immune responses to pathogens and malignant cells can occur in non-lymphoid tissue, where cells actively migrate from the blood stream to peripheral tissues, or within the local tissue-draining lymph node. The migration of specific lymphocyte subsets to peripheral tissues or lymph nodes is regulated by differential expression of adhesion molecules and chemokine/chemokine receptors. Lymphocyte entry into lymph nodes occurs through specialized post capillary high endothelial venules (HEV). Migration through HEV requires overlapping functional interactions between adhesion molecules to capture cells from the blood stream and enable interactions with the endothelium. Upon capture, lymphocytes roll along the venule endothelial wall, are activated by chemokines, undergo firm adhesion, and finally transmigrate through the endothelium. This process, known as the adhesion cascade, results in lymphocyte migration out of the blood and into peripheral lymphoid or non-lymphoid tissue.

L-selectin is an adhesion molecule expressed by all naïve lymphocytes and initiates capture and fast rolling of cells from the blood. The role of L-selectin in lymphocyte migration to peripheral lymph nodes (PLN) has been well documented. The importance of L-selectin in lymphocyte migration into lymph nodes has been demonstrated *in vitro* through reduced adhesion to HEV, and *in vivo* by anti-L-selectin antibody blockade resulting in >90% reduction in migration of lymphocytes to PLN (374). Furthermore, studies using L-selectin-deficient (L-selectin$^{-/-}$) mice demonstrated 70% reduction in PLN cellularity (129), and L-selectin$^{-/-}$ lymphocyte adoptive transfer

assays further demonstrated the requirement for L-selectin-dependent migration into PLN (82).

In addition to L-selectin function in lymphocyte migration to PLNs, L-selectin may also mediate lymphocyte homing to peripheral tissues during inflammation. Endothelial-expressed L-selectin ligands include glycosylation-dependent cell adhesion molecule-1, CD34, Sgp200, podocalyxin, and endomucin. In addition, expression of L-selectin can result in lymphocyte secondary tether formation by binding to the ligands P-selectin glycoprotein ligand-1 (PSGL-1), endoglycan and hematopoetic cell E- and L-selectin ligand (130, 160-163). Thus, L-selectin is required for optimal lymphocyte migration to PLN and may also play a role in migration of lymphocytes to non-lymphoid tissues during inflammation.

The regulatory T (Treg) cell subset of lymphocytes is important for maintaining immunologic homeostasis. The importance of Treg cells has been demonstrated by studies in which depletion of Treg cells resulted in severe autoimmune disease (11), increased graft rejection (12), and persistent infection (375). However, during chronic inflammation such as cancer, Treg cell suppression of immune responses can prevent the clearance of malignant cells, whereas depletion of Treg cells can lead to reduction in tumor growth and metastases (13, 376, 377). As previously demonstrated, Treg cell populations increase during tumor progression within the tumor-draining lymph nodes (dLN) and tumor tissue (Chapter 3, Figs. 13, 19). These findings are consistent with previous studies reporting accumulation of Treg cells at tumor sites and dLNs, leading to impediment in the generation and activation of tumor-specific effector T cell responses to tumor tissues as well as reduced efficacy of immunotherapy (259-267).

Treg cells mediate their suppressive effects through direct cell-cell contact and/or paracrine signaling through the release of anti-inflammatory cytokines such as transforming growth factor beta (TGF-β) and interleukin (IL)-10 (190). Thus, the proximity of Treg cells to their suppressive targets is critical for optimal immune suppression. Specifically, the migration of Treg cells to tumor environments and dLNs where effector cells are located is important for their inhibitory function. In fact, both $CD4^+$ T cells and Treg cells are primed in the same draining lymph nodes during tumor progression (189). Furthermore, Treg cells have been shown to preferentially migrate and accumulate in tumors and ascites (266).

Treg cell migration to PLNs is facilitated by L-selectin. Importantly, although Treg cells and conventional T cells both require L-selectin for migration into resting PLNs, Treg cells showed an ~3-9 fold lower migration rate (192). In addition, L-selectin-dependent homing to peripheral tissues and tissue draining lymph nodes is required for Treg cell activation in allograft models (373, 378, 379). However, the mechanisms that control Treg migration to tumors and dLNs have not been defined.

This study was initiated with the hypothesis that L-selectin directs preferential homing of Treg cells to tumor tissue and dLNs. As expected, Treg cell migration to dLNs was increased in comparison to contra-lateral non-tumor draining lymph nodes (ndLN). Homing of Treg cells specifically to dLNs compared to ndLNs was further amplified as tumors progressed. Importantly, Treg cell migration into dLNs during tumor progression was mediated primarily by L-selectin function. Surprisingly, in mice with late stage tumors, there was an increase in L-selectin-independent migration into dLNs. Furthermore, Treg cell migration into solid tumor tissue increased during tumor growth,

and was mediated, in part by L-selectin in late-stage tumors. However, migrated Treg cell populations did not accumulate within the dLNs or tumor tissue at any stage of tumor progression. Thus, Treg cells preferentially migrate to tumor tissue and dLNs in an L-selectin-dependent manner and this migration is enhanced during tumor progression.

MATERIAL AND METHODS

Animals

Balb/c and Balb/cFoxp3^{EGFP} (Foxp3^{EGFP}) mice (346) were purchased from The Jackson Laboratory (Bar Harbor, ME). The L-selectin null mutation (129) from a C57BL/6 strain background was backcrossed more than 10 generations onto the Balb/c strain background. Balb/c/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) mice were then intercrossed with Foxp3^{EGFP} mice to produce Foxp3$^{EGFP+/+}$L-selectin$^{-/-}$ (Foxp3^{EGFP}/L-selectin$^{-/-}$) homozygous mice. The Foxp3^{EGFP} genotype was assessed by PCR analysis of genomic DNA from ear biopsies and lymphocyte cell-surface expression of L-selectin was assessed by staining of blood leukocytes with the fluorescein isothiocyanate (FITC)-conjugated LAM1-116 mAb (125) and analyzed by flow cytometry. For all studies, female mice 8-12 weeks of age were used. All mice were maintained under specific pathogen-free conditions and screened regularly for pathogens. All studies and procedures were approved by the Animal Care and Use Committee of the University of Wisconsin-Milwaukee.

Murine 4T1 cell line and tumor induction

The 4T1 murine tumor cell line was purchased from ATCC (CRL-2539, Manassas, VA) and used to induce solid tumors in Balb/c, Foxp3^{EGFP}, and Foxp3^{EGFP}/L-selectin$^{-/-}$ female mice. 4T1 cells were maintained in complete RPMI medium (RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, (all from Invitrogen, Gaithersburg, MD) and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Cells were grown in a humidified

incubator supplied with 5% CO_2 at 37ºC to 70% confluence. 4T1 cells were lifted and resuspended ($0.2x10^6$/ml) in supplement-free RPMI medium for injections. Adult female mice (8-12 week old) were injected subcutaneously with $1x10^4$ 4T1 cells in 50 µl in the lower left mammary fat pad. Mice were euthanized by CO_2 asphyxiation 1, 2, 3, or 4-weeks after tumor inoculation.

***In vivo* Migration Assays**

Splenocyte single-cell suspensions from Foxp3^{EGFP} or Foxp3 ^{EGFP}/L-selectin$^{-/-}$ mice were prepared as described (82), filtered through nylon mesh and washed with phosphate-buffered saline (PBS). Erythrocytes were lysed with 0.1 M ammonium chloride solution. Cell numbers were counted using a hemocytometer, and cell counts were adjusted to $12.5x10^6$ cells/ml. Cells were labeled with EZ link-sulfo-NHS-biotin (80 ug/ml, Pierce, Rockford, IL) for 15 min at room temperature. Following labeling, cells were washed with PBS, counted and resuspended at a concentration of 100 x 10^6 cells/ml. Donor spleen cells ($40x10^6$ cells in 400 µl) were injected intravenously (lateral tail vein) into Foxp3 ^{EGFP} tumor-free mice or mice with 4T1 tumors that progressed to 1, 2, 3 or 4 weeks. Two hours or two days after injection, blood was collected, and mice were euthanized. Spleen, tumor, dLN, ndLN, mesenteric lymph nodes (MLN), and Peyer's patches were harvested and single-cell suspensions were prepared.

Lymphocyte Isolation and Flow Cytometry

Control (tumor-free) and 1-, 2-, 3- and 4-week tumor-bearing mice were sacrificed and the blood, spleen, MLN (superior mesenteric cords), tumor, dLN, ndLN and Peyer's patches were harvested for analysis. Single-cell suspensions of tissues were prepared as previously described (82). Briefly, spleen and lymph nodes were minced with

needles and filtered through nylon mesh, and washed twice with PBS. For splenocyte suspensions, erythrocytes were lysed with Tris-buffered 0.1M ammonium chloride solution. Peyer's patches were incubated in Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution supplemented with HEPES (15 μM), FBS (10%; all from Invitrogen) and EDTA (5 μM) for 30 min at 37ºC. Peyer's patches were mechanically dissociated with frosted glass slides, filtered through nylon mesh, and washed twice with PBS. Tumors were harvested, minced with scissors, and incubated for 1 hour at 37ºC in collagenase buffer (20.5 U/μl DNAse, 562 U/ml collagenase A, both from Sigma Aldrich, and 5% [w/v] bovine serum albumin, Thermofisher, Waltham, MA). Transfer pipettes were used to shear-dissociate tumor tissues prior to filtering through nylon mesh, which was followed by washing twice with PBS. Cell counts for all tissues were determined using a hemocytometer. For blood samples, leukocytes were counted after lysis in 2% acetic acid.

Single-cell suspensions of donor splenocytes and all recipient tissues were stained with PE-conjugated anti-mouse CD4 (RM4-5, BD Biosciences, Mountain View, CA) and biotinylated cells were identified using allophycocyanin (APC)-conjugated neutralite-avidin (Southern Biotech, Birmingham, AL). Isotype-matched rat IgG antibodies were used as controls for non-specific staining. Cells with forward and side light scattered properties of mononuclear cells were gated and the total number and frequency of $CD4^+EGFP^-$ and $CD4^+EGFP^+$ cells from each tissue were determined using a FACSCalibur flow cytometer using CellQuest™ Pro software (BD Biosciences); analysis was performed using FlowJo™ analysis software (Tree Star Inc., Ashland, OR). The percentage of migrated cells in each tissue was determined by gating on biotinylated

cells. The total number and frequency of migrated $CD4^+EGFP^-$ and $CD4^+EGFP^+$ cells from each tissue were determined by gating on CD4 and EGFP.

Statistical analysis

All data are presented as mean values ± SEM. Significant differences between sample means were determined using one-way ANOVA and the Student's *t*-test. A paired Student's *t* test was used for comparisons between results of ndLN and dLN within the same animal. A p value of <0.05 was considered to be statistically significant.

RESULTS

Treg cell migration to the spleen is enhanced in during late-stage tumor progression

During tumor progression, Treg cell populations increase in the spleen, tumor and dLNs (Chapter 3). Increases in Treg cell populations may be due to an increase in migration of these cells to tumors and dLNs. To determine the contribution of migration to Treg cell populations in lymphoid tissue and tumors during progressive stages of tumor growth, short-term (2 hour) or long-term (2 day) adoptive transfer assays were performed. Specifically, splenocytes from syngeneic $Foxp3^{EGFP}$ mice were biotinylated and transferred into tumor-free or 4T1 tumor-bearing recipient mice. Transferred Treg cells were allowed to migrate for 2 hours to assess cell migration directly from the blood into lymphoid or tumor tissue. Additionally, to determine lymphocyte retention, or lymphatic entry into secondary lymphoid tissue, adoptively transferred Treg cells were allowed to migrate for 2 days. To compare Treg cell homing patterns, the frequency of migrated cells (percent of injected) in each tissue was analyzed by flow cytometry (Fig. 23A). In general, tumor progression had little-to-no affect on the migration of $CD4^+$ T cell or Treg cell migration within mucosal lymphoid tissues such as MLN and Peyer's patches of wild type or L-$selectin^{-/-}$ mice (data not shown). Therefore, these tissues were excluded from further studies.

Within the spleen, 2 hour migration assays showed that Treg cell migration initially decreased in mice bearing 1-week tumors compared to control mice and significantly declined by 30% in mice bearing 2-week tumors (Fig. 23B). However, Treg cell migration to the spleen returned to control levels in 3-week tumor-bearing mice and

further increased to significantly elevated levels in mice bearing 4-week tumors (by 96%). Interestingly, in 2 day migration assays, migrated Treg cells were only significantly elevated in the spleen of mice bearing 4-week tumors (Fig. 23C). Thus, in mice with late-stage tumors, increased numbers of Treg cells are recruited to the spleen, but migrated cells do not accumulate within the spleen.

Preferential Treg cell recruitment to tumor-draining lymph nodes during tumor progression

Previously, Treg cell populations were shown to selectively increase in the dLNs of mice during tumor progression (Chapter 3). To determine whether this increase in Treg cell population was due to increased recruitment of Treg cells from the blood through HEV into dLNs, short-term adoptive transfer assays were performed. Indeed, after 2 hours, Treg cell migration to the dLNs in mice bearing 2-, 3- and 4-week tumors was significantly increased compared to both tumor-free (by 3.7-, 5.6-, and 5.7-fold, respectively) and 1-week tumor-bearing mice ($p<0.05$, Fig. 24A). Treg cell migration to dLNs was also enhanced in long-term migration assays (Fig. 24B). In fact, Treg cell migration to dLNs was significantly greater after 2 days of migration in mice bearing 1-, 3- and 4-week tumors (by 2.6-, 3.6-, and 2.5-fold, respectively, $p<0.05$) compared to controls. Surprisingly, except in mice bearing 1-week tumors, the number of migrated Treg cells did not significantly increase in 2 day migrations compared to 2 hour assays, indicating that while increases in Treg cell migration to dLNs is correlated with tumor progression, their accumulation is not. Thus, Treg cell migration from the blood contributes to an influx of Treg cells into dLNs during tumor progression.

By contrast, Treg cell migration from the blood into ndLNs did not change during tumor progression (Fig. 24). Consistent with stable Treg cell numbers during tumor progression (Chapter 3, Fig. 14), Treg cells showed no preferential migration into ndLNs, nor did Treg cells accumulate during tumor progression. Furthermore, short-term Treg cell migration from the blood into dLNs was significantly greater compared to ndLNs in mice bearing 2-, 3-, and 4-week tumors (by 5.8-, 4.0- and 3.2-fold, respectively, Fig. 24). Similarly, Treg cell recruitment to dLNs was significantly greater than ndLNs after 2 days in mice bearing 3-week (by 48%) and 4-week tumors (by 49%). Therefore, Treg cells preferentially migrate to dLNs compared to ndLNs during tumor progression.

Treg cell migration into tumor tissue is enhanced in late stage tumors

Treg cells are immunologic suppressors, and enable tumors to evade anti-tumor immune responses. Treg cell suppression is mediated by cell-cell contact or by paracrine secretion of anti-inflammatory cytokines. Therefore, the location and proximity of Treg cells to target cells is critical for their suppressive activity. As previously demonstrated, the number of Treg cells within tumor tissue steadily increased during 4T1 tumor progression (Chapter 3, Fig. 19). However, whether this accumulation is due to active homing of Treg cells into the tumor, increased proliferation of Treg cells or differentiation of Treg cells from naïve T cells is not clear. Therefore, the ability of Treg cells to migrate into 4T1 tumors was assessed in mice with advancing stages of tumor progression (Fig. 25A). Treg cell migration from the blood into tumor tissue increased steadily as tumors progressed (Fig. 25B). Specifically, compared to 1-week tumor-bearing mice, Treg cell migration increased by 17-fold in mice bearing 2-week tumors,

and further increased in mice bearing 3-week tumors (by 44-fold) and 4-week tumors (by 83-fold) in short-term migration assays. However, while Treg cell migration into 1-week tumor-bearing mice could now be detected in long-term migration assays, there was no increase in Treg cell migration in mice bearing 2-4-week tumors in contrast to short-term migration assays. Interestingly, the presence of very few migrated Treg cells in tumor tissues from mice bearing 2- and 3-week tumors, compared to short-term assays, suggests that these cells either migrated out of the tissue or underwent apoptosis. Furthermore, the finding that mice with tumors progressed to 4 weeks had similar levels of migrated Treg cells in both 2 hour and 2 day migration assays demonstrates that these cells do not accumulate within the tumor. Therefore, Treg cells actively migrate from the blood into tumor tissue but this migration alone is unlikely to account for accumulation of these cells within the tumor as the disease progresses.

Treg cell migration to the spleen during 4T1 tumor progression is increased in L-selectin$^{-/-}$ mice

Lymphocyte migration and recirculation through peripheral and lymphoid tissue is regulated through expression of adhesion molecules present on lymphocytes that bind to ligand on the endothelium. L-selectin is required for almost all lymphocyte and Treg cell entry into lymph nodes, and is important in lymphocyte migration to peripheral tissues where L-selectin ligands are expressed. However, the role of L-selectin in Treg cell migration to secondary lymphoid tissue, blood, and solid tumor tissue in mice during tumor progression has not been defined. Therefore, to determine the role of L-selectin in Treg cell migration and accumulation during tumor progression, Foxp3^{EGFP}/L-selectin$^{-/-}$

mice were used. Foxp3^{EGFP}/L-selectin$^{-/-}$ splenocytes were biotinylated and adoptively transferred to tumor-free or Foxp3^{EGFP} mice bearing 1-, 2-, 3- or 4-week tumors. L-selectin$^{-/-}$ splenocytes were allowed to migrate for 2 hours or 2 days before lymphoid and tumor tissues were harvested.

Within the spleen, the number of transferred L-selectin$^{-/-}$ Treg cells found after 2 hours of migration steadily increased during tumor progression with 2.2-, 3.3- and 3.8-fold increases being observed in mice with 2-, 3-, and 4-week tumors, respectively, compared to control mice (Fig. 26). After 2 days of migration, Treg cell recruitment to the spleen was also increased in 2- and 4-week tumor-bearing mice (Fig. 26B), but were similar to levels seen in 2 hour migration assays, suggesting that L-selectin$^{-/-}$ Treg cells were entering at a faster rate but not retained within the spleen during tumor progression.

L-selectin is required for Treg cell migration from the blood into tumor-draining lymph nodes

Migration of lymphocytes into resting PLN is dependent on L-selectin expression. However, whether L-selectin expression is required for Treg cell migration into activated dLNs has not been described. Two hours following transfer, very few L-selectin$^{-/-}$ Treg cells were able to migrate from the blood into dLNs at any stage of tumor progression (Fig. 27A). By contrast, Treg cells were able to migrate to dLNs after 2 days, and their recruitment increased with tumor progression (Fig. 27B). Specifically Treg cell recruitment after 2 days of migration was significantly enhanced in mice bearing 2-week (by 3.8-fold), 3-week (by 4.7-fold) and 4-week tumors (by 5.2-fold) compared to controls. Furthermore, Treg cell recruitment was specific to dLNs, as demonstrated by

finding no significant difference in migration of Treg cells to ndLNs at any stage of tumor progression (Fig. 27). While L-selectin$^{-/-}$ mice showed no significant increase in migration from the blood to dLNs during tumor progression compared to controls in short-term migration assays, the number of migrated Treg cells was significantly higher compared to ndLNs within the same tumor-bearing animal. In fact, compared to ndLNs, L-selectin$^{-/-}$ Treg cell migration from the blood was 3.2-fold higher in mice bearing 3-week tumors and 7.2-fold higher in mice bearing 4-week tumors (Fig. 27). Consistent with an increase in Treg cell migration to dLNs after 2 hours, dLN specific recruitment of Treg cells was even more pronounced after 2 days, by 2.7-, 4.8-, and 8-fold in mice bearing 2-, 3- and 4-week tumors, respectively, compared to ndLNs (Fig. 27). Thus, low numbers of Treg cells can specifically migrate to dLNs in the absence of L-selectin, suggesting other mechanisms of recruitment exist.

L-selectin$^{-/-}$ Treg cells migrate into late stage 4T1 tumor tissue

Treg cells have been shown to be elevated within tumors and have been correlated to poor prognosis in patients with cancer while depletion has resulted in anti-tumor immunity (261, 380). We previously demonstrated that Treg cell populations increase in 4T1 tumor tissue during tumor progression (Chapter 3, Fig. 19). However, whether Treg cell migration to tumors contributes to increases in Treg cell populations, and the mechanisms that control their migration, have not been defined. In 2 hour migration assays, small numbers of L-selectin$^{-/-}$ Treg cells were able to migrate into tumor tissue only in late stage tumors. Specifically, compared to 1 week tumors, L-selectin$^{-/-}$ Treg cell migration to late stage 3 and 4 week tumors was significantly increased (by 9-fold and

8.8-fold, respectively, Fig. 28A). However, L-selectin$^{-/-}$ Treg cells did not accumulate within the tumor, as demonstrated by similar numbers of cells being found after 2 hour and 2 day migration assays (Fig. 28B). Thus, some Treg cell entry can occur into late stage tumors in the absence of L-selectin.

L-selectin deficiency enhances Treg cell recruitment to the spleen

In general, L-selectin$^{-/-}$ Treg cell recruitment to the spleen was enhanced compared to wild type Treg cells during tumor progression. Specifically, compared to Foxp3^{EGFP} mice, L-selectin$^{-/-}$ Treg cell migration to the spleen was increased in mice bearing 2- and 3-week tumors (by 2-fold, Fig. 29). In 2 day migration assays, L-selectin$^{-/-}$ Treg cell migration to the spleen was increased in tumor-free mice (by 2.9-fold), as previously described for other T cell populations with L-selectin deficiency on the C57BL/6 background (82, 129). Treg cell recruitment to the spleen after 2 days was enhanced in mice bearing 1-week (by 2.2-fold), 2-week by 2.5-fold), and 3-week tumors (by 1.8-fold). Therefore, L-selectin deficiency resulted in an increased migration of Treg cells to the spleen throughout tumor progression, suggesting that these cells are unable to migrate into other peripheral sites.

L-selectin is required for optimal Treg cell migration into tumor-draining and non-tumor draining lymph nodes

As shown above (Fig. 25), some increased migration of Treg cells into dLNs was observed in the absence of L-selectin expression. To determine the degree to which L-selectin mediates Treg cell migration, the levels of wild type and L-selectin$^{-/-}$ Treg cell migration into dLNs were compared. Treg cells from L-selectin$^{-/-}$ mice showed severe

reductions in migration from the blood into dLNs compared to Treg cells from wild type donors (Fig. 30). In tumor free mice, short-term Treg cell migration into lymph nodes tended to be greater than L-selectin$^{-/-}$ Treg cell migration ($p=0.055$). During tumor progression, short-term migration of wild type Treg cell migration from the blood into dLNs was dramatically greater than L-selectin$^{-/-}$ Treg cells at all time points (by 80-95%). Similarly, in long-term migration assays, compared to L-selectin$^{-/-}$ Treg cells, wild type Treg cell migration was 19-fold greater in tumor-free mice, and remained significantly elevated in mice bearing 1-, 2-, 3- and 4-week tumors (by 29-, 9.9-, 14.6- and 8.9-fold, respectively). Interestingly, the greatest difference between L-selectin$^{-/-}$ and wild type Treg cell migration occurred in mice with early stage tumors, and this difference decreased in later stage tumors, as L-selectin$^{-/-}$ Treg cell migration increased. Therefore, L-selectin is required for optimal Treg cell migration into dLNs during 4T1 tumor progression, but some L-selectin-independent entry occurs later in disease.

L-selectin is required for Treg cell migration from the blood into late stage 4T1 tumors

Interestingly, L-selectin was important not only for lymphocyte migration into dLNs, but also into tumor tissue. Specifically, while L-selectin$^{-/-}$ Treg cells could migrate into the tumor in short-term assays, they did so at reduced numbers (by 3.3-fold, Fig. 31) in 4-week tumors. Similarly, L-selectin$^{-/-}$ Treg cell migration into 4-week tumors during long-term migration assays tended to be decreased, but did not reach significance. Therefore, L-selectin plays a role in mediating optimal Treg cell migration into tumor

tissue during late stage tumor progression, and thus may account for some of the decrease in Treg cells observed in tumors of L-selectin$^{-/-}$ mice.

DISCUSSION

Treg cells mediate immune suppression through secretion of anti-inflammatory cytokines and effector cell inhibition. In cancer, Treg cells suppress anti-tumor immune responses, allowing tumors to escape immune-mediated clearance. We previously demonstrated that during 4T1 tumor progression, Treg cell populations increase in the spleen, tumor, dLNs, and the blood (Chapter 3). However, this increase could be due to proliferation of Treg cells within these tissues, induction of Treg cells from naïve T cells into induced Treg cells (iTreg), or an influx via migration of natural Treg (nTreg) cells to these tissues. Therefore, we sought to determine if Treg cells specifically migrate to tumors and dLNs using adoptive transfer assays. Indeed, Treg cell migration was specifically increased in dLNs, compared to contralateral ndLNs. Furthermore, Treg cell migration increased during tumor progression, indicating Treg cell-specific homing to dLNs (Fig. 24). Treg cells also migrated into tumor tissue, and an increase in migration was correlated with tumor progression (Fig. 25).

Specific lymphocyte homing to peripheral lymphoid tissue and sites of inflammation is regulated by adhesion molecules present on lymphocytes that bind to ligands on endothelial cells. L-selectin is essential for optimal lymphocyte migration into lymph nodes, and also plays a role in migration of lymphocytes to sites of inflammation and tumors (80, 120, 381). Treg cells have been shown to express high levels of L-selectin, which directs their migration into resting lymph nodes (192). However, the role of L-selectin in Treg cell migration to activated dLNs and tumor tissue has not been defined. Therefore, we hypothesized that L-selectin is required for Treg cell homing to dLNs and tumor tissue. Importantly, L-selectin was required for optimal Treg cell

migration from the blood into dLNs (Fig. 30). In addition, L-selectin facilitated Treg cell migration into solid tumor tissues during late stages of disease (Fig. 31).

In this study, we employed adoptive transfer assays to determine Treg cell migration into tissue directly from the blood using short-term assays. Treg cell entry into lymph nodes through HEV can occur within 5-10 minutes, while lymphocyte transit through a lymph node takes between 12 and 24 hours (382, 383). Therefore, using 2 hour assays, we could determine specific migration of Treg cell from the blood into lymphoid tissue. Alternatively, lymphocytes can exit the blood stream and enter peripheral tissue, and drain into lymph nodes through afferent lymphatics (384-386). Indeed, lymphocyte migration from the blood to lymph through peripheral tissues such as the skin is highly dynamic with a transit time peaking at approximately 24 hours (387). To determine lymphocyte entry into dLNs via lymphatics, donor L-selectin$^{-/-}$ cells and 2 day migration assays were used to allow migration through tumor tissue. In addition, long-term migration assays enable determination of specific lymphocyte retention within secondary lymphoid and tumor tissues. Most strikingly, while the vast majority of Treg cell migration to dLNs was dependent on L-selectin, low numbers of L-selectin$^{-/-}$ Treg cells were able to enter later during tumor progression in 2 day migration assays (Fig. 27), demonstrating L-selectin independent entry into dLNs. This was most likely due to Treg cell entry via afferent lymphatics that drain the tumor tissue. This is supported by a lack of L-selectin$^{-/-}$ Treg cell entry into ndLNs in 2 day migration assays, where lymphatic drainage from a tumor is absent (Fig. 27). However due to poor lymphatic drainage of tumor tissue, L-selectin-independent Treg cell migration through tumor-draining afferent lymphatics accounted for only a small proportion of Treg cell migration into the dLNs.

Surprisingly, while L-selectin$^{-/-}$ Treg cells were increased in the dLNs of 2-week tumor mice after 2 day migration assays, there was no significant increase in Treg cells within the tumor tissue in 2-week tumor-bearing mice. However, it is possible that Treg cells may be within peri-tumoral tissue surrounding the developing tumor, and drain into the dLNs in early stage tumors. In fact, Treg cells have been shown to be increased in peri-tumoral tissue in breast cancer patients (388, 389). Lymphatic drainage of peri-tumoral tissue may be more efficient than tumor lymphatics, similar to that for normal lymphatic drainage of the skin, leading to a greater number of Treg cells entering through afferent lymphatics into dLNs. Alternatively, it is possible that L-selectin-independent Treg cell migration may also occur from the blood through the HEV into dLNs, where in wild type mice the proportion of L-selectin-independent Treg cell migration may be too low to detect. After 2 days of migration, small numbers of L-selectin$^{-/-}$ Treg cells migrating through the HEV could account for this increase within dLNs. However, L-selectin$^{-/-}$ Treg cell migration into ndLNs did not change after 2 day migrations, suggesting that L-selectin-independent entry into lymph nodes through HEV is not the case, or that dLNs have altered HEV morphology and/or adhesion molecule expression profiles.

Interestingly, while previous studies examining CD4^{+} T cell migration to lymph nodes reported an increase in the number of migrated cells in long-term migration assays compared to the number of migrated cells in short-term migration assays (390), this was not the case for Treg cell migration in 4T1 tumor-bearing mice. Indeed, this trend holds true for tumor and ndLNs of tumor-free mice injected with wild type Treg cells and both wild type and L-selectin$^{-/-}$ Treg cell migration into MLN (data not shown). This suggests that in tumor-free mice, migrated wild type Treg cells accumulate within the lymph nodes

after 2 days; but that during tumor progression, Treg cells enter and exit lymph nodes at a similar rate, or undergo apoptosis within the lymph node.

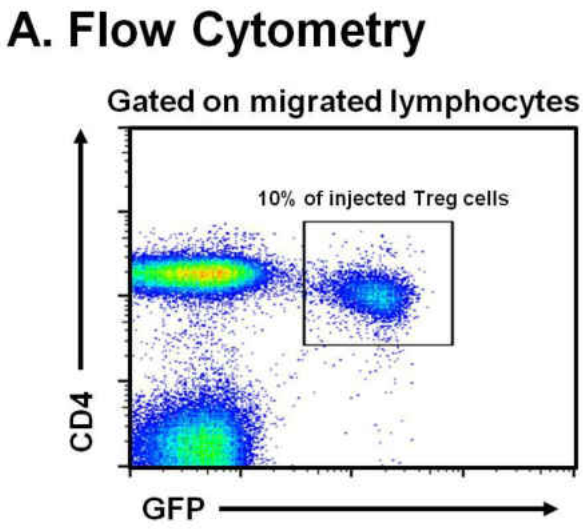
Increased L-selectin$^{-/-}$ Treg cell migration compared to wild type Treg cell migration into the spleen can be accounted for by the requirement of L-selectin in Treg cell migration into peripheral lymphoid tissues. Both wild type and L-selectin$^{-/-}$ Treg cell migration to the spleen increased during tumor progression. However, no further retention after 2 days suggests that recirculating Treg cells are entering and exiting at the same rate, or Treg cells accumulate but undergo apoptosis within the spleen.

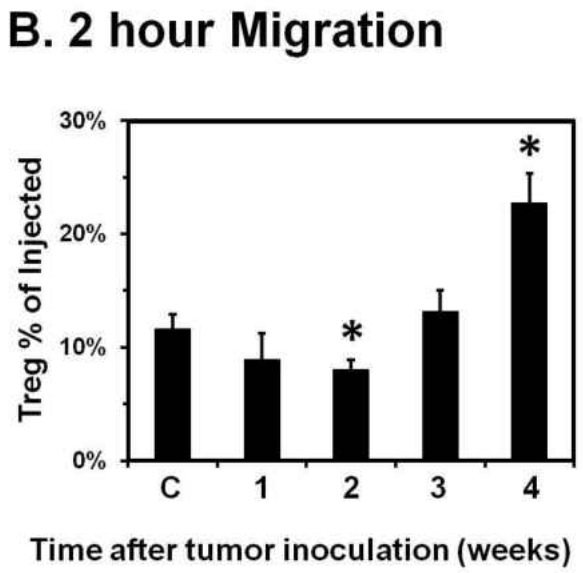
Importantly, Treg cell migration into 4-week tumors was partly dependent on expression of L-selectin (Fig. 31). Treg cell migration into solid tumor tissue increased steadily during tumor progression, while L-selectin$^{-/-}$ Treg cell migration did not increase between 3- and 4-week tumor-bearing mice. This suggests that in late stage tumors, expression of L-selectin is required for optimal Treg cell infiltration into tumors. L-selectin-dependent migration into tumors may be through capture of Treg cells via secondary tethers by binding to PSGL-1 expressed on the surface of other leukocytes (162). Importantly, L-selectin expression can also mediate tissue specific homing by binding to vascular endothelium-expressed ligands such as glycosylation-dependent cell adhesion molecule-1, CD34, Sgp200, podocalyxin, and endomucins (130). Furthermore, lymphocyte migration into tumors may depend on synergistic and cooperative interactions between L-selectin function and other adhesion molecules. Inflamed tumor vascular endothelial expression of intercellular cell adhesion molecule-1 (ICAM-1) and/or vascular cell adhesion molecule-1 (VCAM-1) may mediate Treg cell migration through integrin binding. In fact, it has been demonstrated that L-selectin function

cooperates with lymphocyte function associated antigen-1 (LFA-1) interactions with ICAM-1 and optimizes leukocyte/endothelial interactions and migration into sites of inflammation (119, 120). L-selectin has also been shown to cooperate with $\alpha_4\beta_7$ integrin/mucosal addressin cell adhesion molecule-1 (MAdCAM-1) interactions in lymphocyte migration to gut associated lymphoid tissue where MAdCAM-1 is expressed (127, 128). Furthermore, L-selectin functions cooperatively with $\alpha_4\beta_7$ integrin/VCAM-1 interactions to facilitate increased Treg cell adhesion *in vitro* (Chapter 2). Importantly, VCAM-1 is upregulated on inflamed endothelium in response to inflammatory cues (170, 171). Therefore, Treg cell-expressed L-selectin may function cooperatively with other adhesion molecules to optimize migration into tumor tissue.

In this study, we have demonstrated Treg cells have a propensity to specifically home to tumors and dLNs, and this migration is enhanced during tumor progression. Treg cell migration into dLNs occurs primarily from the blood through the HEV and is dependent on L-selectin expression. Furthermore, L-selectin contributes to Treg cell migration into late stage tumor tissue. Therefore, L-selectin-dependent Treg cell migration contributes to an increase in Treg cell numbers within the tumor and dLNs during tumor progression, thereby generating an immunosuppressive environment. Here, we have demonstrated a mechanism that promotes an increase in Treg cell populations during cancer. Elucidating the mechanisms of Treg cell migration to tumor tissue and dLNs may lead to a better understanding of tumor immune evasion and provide new targets for immunotherapeutic strategies.

Figure 23. Treg cell migration to the spleen increases during 4T1 tumor progression. Splenocytes from Foxp3^{EGFP} mice were biotinylated and adoptively transferred into tumor-free or 4T1 tumor-bearing Foxp3^{EGFP} mice and allowed to migrate for 2 hours or 2 days. Recipient tissues were harvested and the percentage of the injected Treg cells were analyzed by flow cytometry. A) Representative flow cytometry analysis of migrated Treg cells from a 2 hour migration in a tumor-free mouse. Lymphocyte populations were gated based on forward and side scatter properties, and further gated on avidin^{+} cells. B) The percentage of injected Treg cells that migrated to the spleen after 2 hour migration assays in tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. C) The percentage of injected Treg cells that migrated to the spleen after 2 day migration assays. *$p<0.05$ vs. tumor-free mice. Results represent means ± SEM from 3-8 independent experiments per time point.


A. Flow Cytometry
Gated on migrated lymphocytes
10% of injected Treg cells
CD4
GFP


B. 2 hour Migration
Treg % of Injected
30%
20%
10%
0%
C
1
2
3
4
*
*
Time after tumor inoculation (weeks)

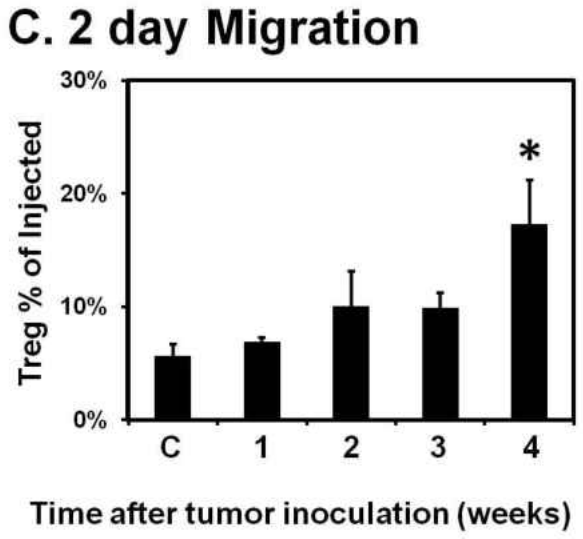

C. 2 day Migration
Treg % of Injected
30%
20%
10%
0%
C
1
2
3
4
*
Time after tumor inoculation (weeks)

Figure 23

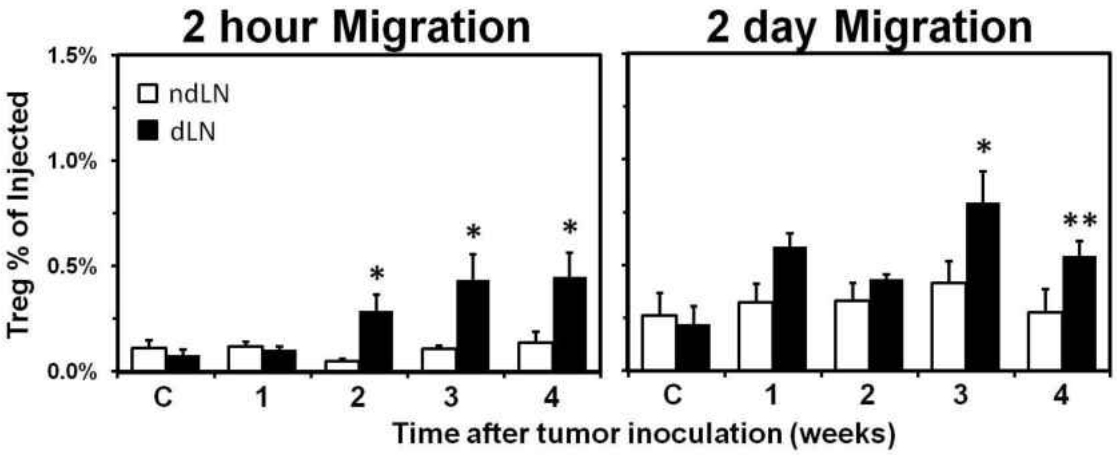


Figure 24. Treg cells preferentially migrate to tumor-draining lymph nodes during 4T1 tumor progression.

Splenocytes from Foxp3^{EGFP} mice were biotinylated and adoptively transferred into tumor-free or 4T1 tumor-bearing Foxp3^{EGFP} mice and allowed to migrate for 2 hours or 2 days. Recipient tissues were harvested and percentage of the injected Treg cells in each tissue was analyzed by flow cytometry. A) The percentage of injected Treg cells that migrated to the non-tumor draining lymph nodes (ndLN) or tumor-draining lymph nodes (dLN) after 2 hour migration assays in tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. B) The percentage of injected Treg cells that migrated to ndLNs and dLNs after 2 day migration assays. For tumor-free mice, left inguinal (ndLN) and right inguinal lymph nodes (dLN) were compared. Results between ndLN and dLN were compared using a paired Student's *t*-test, *$p<0.05$, **$p<0.01$ vs. ndLN. Results represent means ± SEM from 3-8 independent experiments per time point.

Figure 25. Treg cell migration to tumor tissue increases during 4T1 tumor progression.

Splenocytes from Foxp3^{EGFP} mice were biotinylated and adoptively transferred into tumor-free or 4T1 tumor-bearing Foxp3^{EGFP} mice and allowed to migrate for 2 hours or 2 days. Recipient tissues were harvested and percentage of the injected Treg cells in each tissue was analyzed by flow cytometry A) Representative flow cytometry analysis of migrated Treg cells from a 2 hour migration in a 1-week tumor-bearing mouse. Lymphocyte populations were gated based on forward and side scatter properties, and further gated on avidin^{+} cells. B) The percentage of injected Treg cells that migrated to 4T1 tumor tissue after 2 hour migration assays in mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. C) The percentage of injected Treg cells that migrated to the tumor tissue after 2 day migration assays. *$p<0.05$, **$p<0.01$ vs. 1-week tumor-bearing mice. Results represent means ± SEM from 3-8 independent experiments per time point.

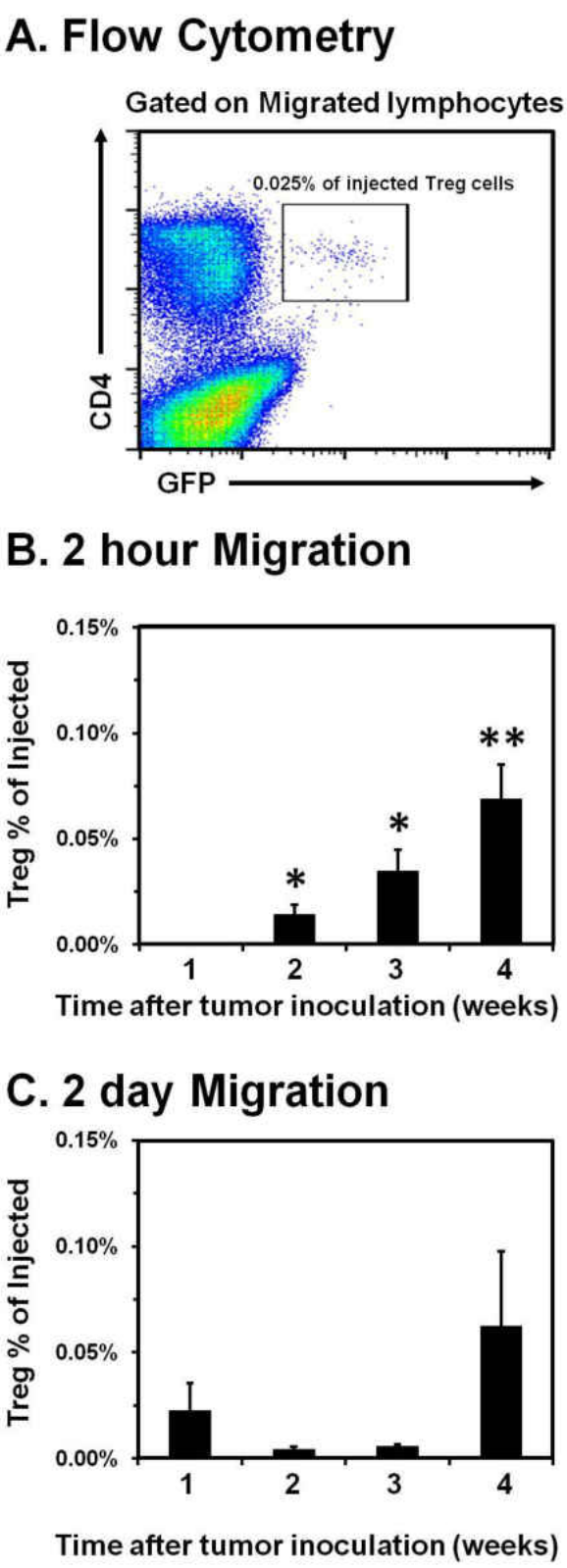

A. Flow Cytometry
Gated on Migrated lymphocytes
0.025% of injected Treg cells
CD4
GFP
B. 2 hour Migration
Treg % of Injected
0.15%
0.10%
0.05%
0.00%
*
*
**
1
2
3
4
Time after tumor inoculation (weeks)
C. 2 day Migration
Treg % of Injected
0.15%
0.10%
0.05%
0.00%
1
2
3
4
Time after tumor inoculation (weeks)

Figure 25

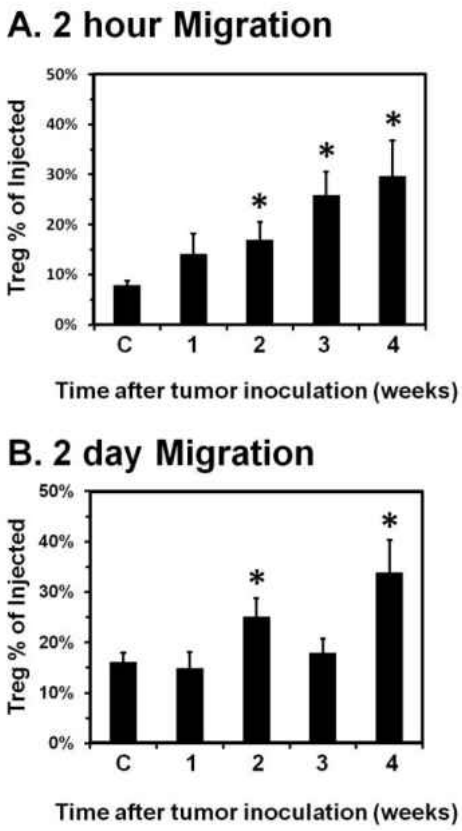


Figure 26. L-selectin$^{-/-}$ Treg cell migration to the spleen increases during 4T1 tumor progression.

L-selectin$^{-/-}$ splenocytes from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice were biotinylated and adoptively transferred into tumor-free or 4T1 tumor-bearing Foxp3^{EGFP} mice and allowed to migrate for 2 hours or 2 days. Recipient tissues were harvested and percentage of the injected Treg cells in each tissue was analyzed by flow cytometry. A) The percentage of injected L-selectin$^{-/-}$ Treg cells migrated to the spleen after 2 hour migration assays tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. B) The percentage of injected L-selectin$^{-/-}$ Treg cells that migrated to the spleen after 2 day migration assays. *$p<0.05$ vs. tumor-free mice. Results represent means ± SEM from 3-7 independent experiments per time point.

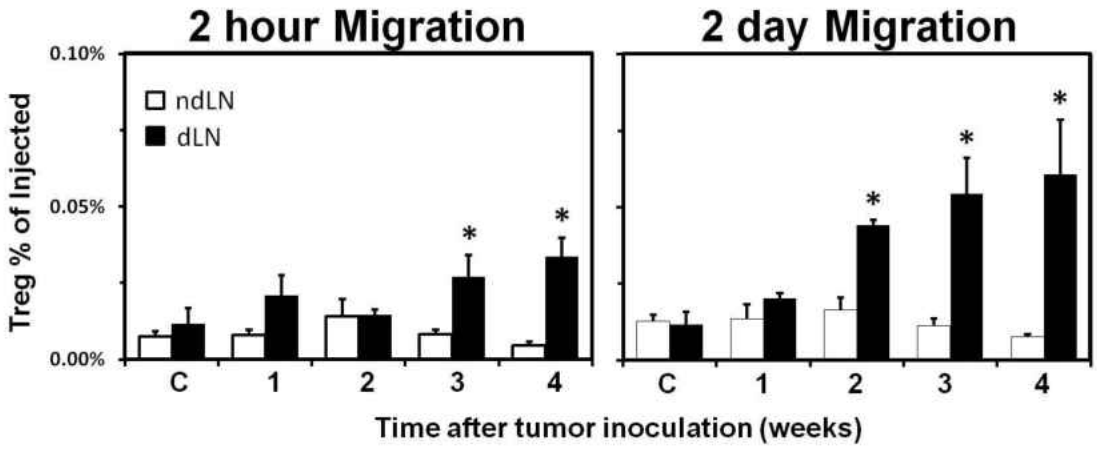


Figure 27. L-selectin$^{-/-}$ Treg cells preferentially migrate to tumor-draining lymph nodes increase during 4T1 tumor progression.

L-selectin$^{-/-}$ splenocytes from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice were biotinylated and adoptively transferred into tumor-free or 4T1 tumor-bearing Foxp3^{EGFP} mice and allowed to migrate for 2 hours or 2 days. Recipient tissues were harvested and percentage of the injected L-selectin$^{-/-}$ Treg cells in each tissue was analyzed by flow cytometry A) The percentage of injected L-selectin$^{-/-}$ Treg cells that migrated to the non-tumor draining lymph nodes (ndLN) or tumor-draining lymph nodes (dLN) after 2 hour migration assays in tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. B) The percentage of injected L-selectin$^{-/-}$ Treg cells that migrated to the dLNs after 2 day migration assays. For tumor-free mice, left inguinal (ndLN) and right inguinal lymph nodes (dLN) were compared. Results between ndLN and dLN were compared using a paired Student's *t*-test. *$p<0.05$ vs. ndLN. Results represent means ± SEM from 3-8 independent experiments per time point.

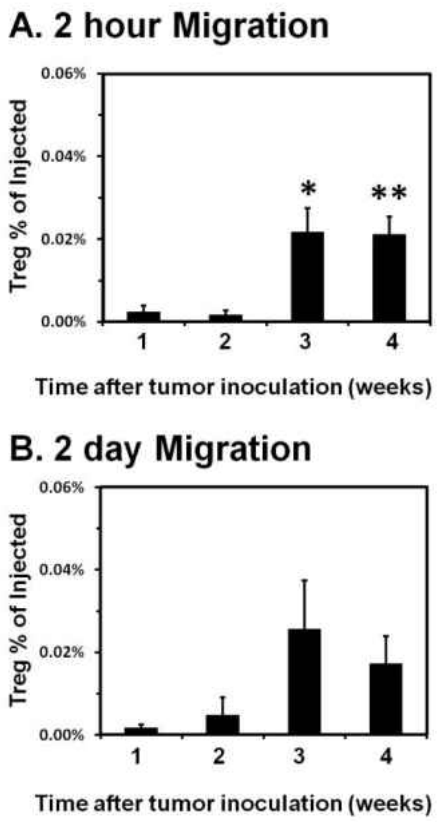


Figure 28. L-selectin$^{-/-}$ Treg cell migration to tumor tissue increases during late stages of 4T1 tumor progression.

L-selectin$^{-/-}$ splenocytes from Foxp3^{EGFP}/L-selectin$^{-/-}$ mice were biotinylated and adoptively transferred into tumor-free or 4T1 tumor-bearing Foxp3^{EGFP} mice and allowed to migrate for 2 hours or 2 days. Recipient tissues were harvested and percentage of the injected Treg cells in each tissue was analyzed by flow cytometry. A) The percentage of injected L-selectin$^{-/-}$ Treg cells that migrated to 4T1 tumor tissue after 2 hour migration assays in mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. B) The percentage of injected L-selectin$^{-/-}$ Treg cells that migrated to the tumor tissue after 2 day migration assays. *$p<0.05$, **$p<0.01$ vs. 1 week tumors. Results represent means ± SEM from 3-8 independent experiments per time point.

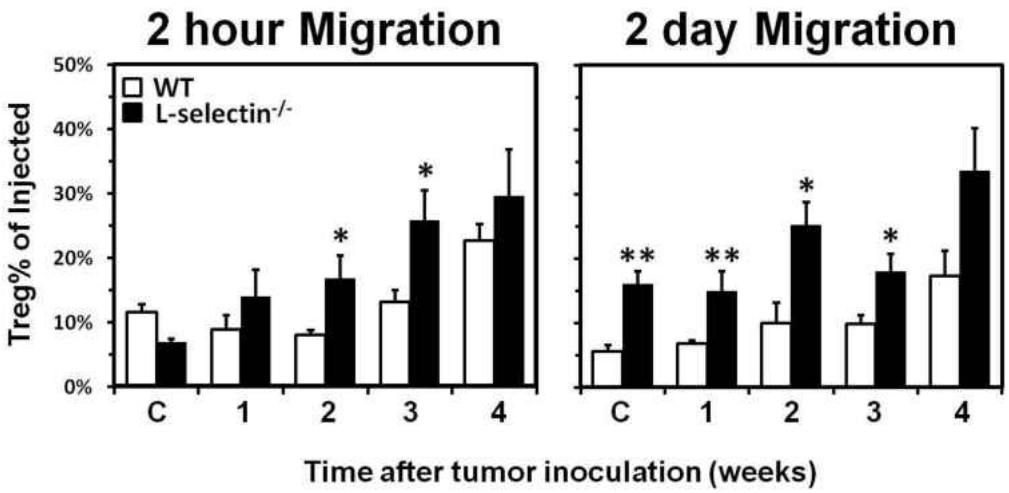


Figure 29. L-selectin deficiency increases Treg cell migration to the spleen during 4T1 tumor progression.

Comparison of the results shown in Figs. 23 and 26. Foxp3^{EGFP} (WT) and Foxp3^{EGFP}/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) Treg cell migration to the spleen was directly compared for 2 hour (*left*) and 2 day migration assays (*right*) in tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. *$p<0.05$, **$p<0.01$ wild type vs. L-selectin$^{-/-}$ Treg cell migration. Results represent means ± SEM from 3-8 independent experiments per time point.

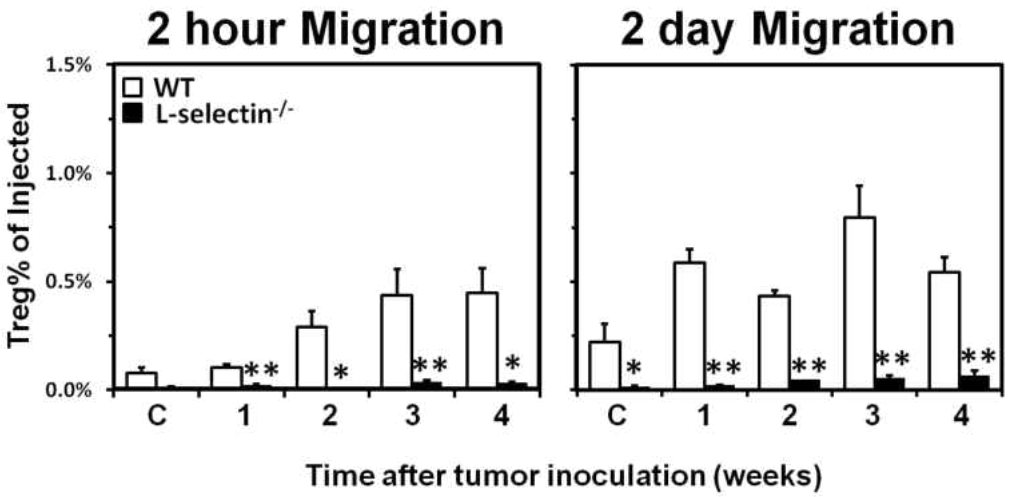


Figure 30. L-selectin is required for optimal Treg cell migration to tumor-draining lymph nodes.

Comparison of the results shown in Figs. 23 and 26. Foxp3^{EGFP} (WT) and Foxp3^{EGFP}/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) Treg cell migration to the left inguinal tumor-draining lymph nodes was directly compared for 2 hour (*left*) and 2 day migration assays (*right*) in tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. *$p<0.05$, **$p<0.01$ wild type vs. L-selectin$^{-/-}$ Treg cell migration. Results represent means ± SEM from 3-8 independent experiments per time point.

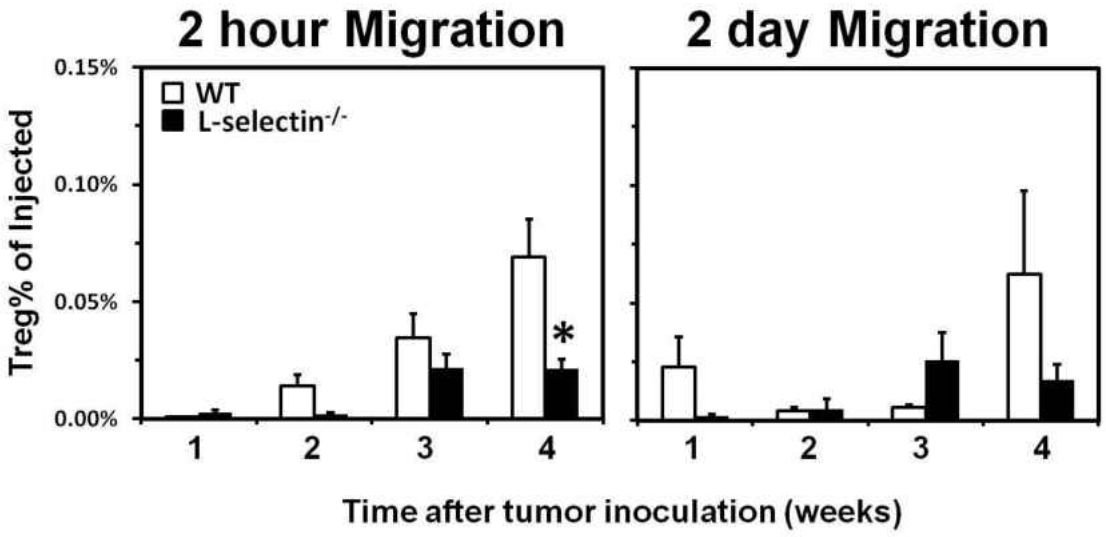


Figure 31. L-selectin is required for optimal Treg cell migration to late stage 4T1 tumors.

Comparison of the results shown in Figs. 23 and 26. Foxp3^{EGFP} (WT) and Foxp3^{EGFP}/L-selectin$^{-/-}$ (L-selectin$^{-/-}$) Treg cell migration to the spleen was directly compared for 2 hour (*left*) and 2 day migration assays (*right*) in tumor-free control (C) mice and mice with 4T1 tumors progressed to 1, 2, 3, or 4 weeks. *$p<0.05$ wild type vs. L-selectin$^{-/-}$ Treg cell migration. Results represent means ± SEM from 3-8 independent experiments per time point.

CHAPTER 5

CONCLUSIONS

CONCLUSIONS

Regulatory T cells (Treg) are a subset of lymphocytes that function to suppress immune responses. Treg cells play a vital role in maintaining immunologic homeostasis and resolving inflammation after pathogens have been cleared or tissues have been repaired. However, Treg cells also function to dampen immune responses to malignant cells, thereby providing tumor immune evasion. Treg cells mediate suppressive function through local secretion of anti-inflammatory cytokines and through cell-cell contact with effector cells. Thus, proximity of Treg cells to their suppressive target is essential to their function. Treg cells have been shown to be elevated within tumor microenvironments, and tumor-draining lymph nodes, but their origin remains ambiguous. Herein, we have described a source of Treg cell increases during tumor progression and further provide one mechanism responsible for Treg cell migration during tumor progression.

The most striking observation from this study was the requirement for L-selectin in Treg cell migration during 4T1 tumor progression. First, we demonstrated that L-selectin functions cooperatively to enhance $\alpha_4\beta_7$ integrin binding to VCAM-1. VCAM-1 is upregulated by endothelium in response to inflammatory cues, and while $\alpha_4\beta_7$ integrin can support lymphocyte adhesion to VCAM-1, this only occurs under conditions of low shear stress. However, at physiologic shear stress $\alpha_4\beta_7$ integrin is not able to bind VCAM-1 without L-selectin function. Importantly, Treg cells express high levels of both L-selectin and $\alpha_4\beta_7$ integrin, which may direct their homing to sites of inflammation. Second, we further demonstrated that L-selectin function cooperates with $\alpha_4\beta_7$ integrin/VCAM-1 interactions to enhance Treg cell adhesion at physiologic shear stress in vitro. However, Treg cells were still able to adhere to VCAM-1-expressing endothelial

cells, in the absence of L-selectin or $\alpha_4\beta_7$ integrin function. This is most likely due to Treg cell subsets that express $\alpha_4\beta_1$ integrin, which binds with high affinity to VCAM-1 and can mediate both capture and adhesion of lymphocytes under shear (222, 339). However, even potential $\alpha_4\beta_1$ integrin-mediated adhesion to VCAM-1 was reduced in the absence of L-selectin function.

In addition to L-selectin function in Treg cell rolling and adhesion *in vitro*, L-selectin is important for Treg cell distribution during tumor progression. Specifically, Treg cells were preferentially increased in tumors and tumor-draining lymph nodes in mice with 4T1 tumors. This increase was specific to Treg cells, whereas $CD4^+$ T cell populations were not as robustly elevated. In the absence of L-selectin, Treg cell increases in tumor-draining lymph nodes were severely reduced, but to a lesser extent than conventional $CD4^+$ T cells, indicating intrinsic difference between these populations, and demonstrating that during tumor progression preferential accumulation of Treg cells leads to immunosuppressive environments within tumors and tumor-draining lymph nodes. Furthermore, L-selectin deficiency led to a drastic decrease in tumor infiltrating Treg cells in late stages of tumor progression. Regardless, in the absence of L-selectin, Treg cells still modestly increased in tumors and tumor-draining lymph nodes during tumor progression, and were significantly higher than early stage tumors or contra-lateral non-tumor-draining lymph nodes. Thus, Treg cells preferentially increase in tumors and tumor-draining lymph nodes creating a more suppressive environment, and L-selectin is required for optimal Treg cell accumulation within these tissues.

Migration of Treg cells to tumors and tumor-draining lymph nodes contributed to an increase in Treg cell populations within these tissues. Treg cells have a propensity to

migrate to tumors and tumor-draining lymph nodes, and their migration was enhanced during tumor progression. Migration into tumor-draining lymph nodes occurs primarily from the blood through HEV and is dependent on L-selectin. However, in the absence of L-selectin, Treg cells were still able to migrate into the tumor-draining lymph nodes from the tumor through afferent lymphatics, suggesting entry through this route is L-selectin independent. In addition, Treg cell entry into tumors is, in part, dependent on L-selectin in late stage tumors. L-selectin-dependent migration into non-tumor-draining lymph nodes is also evident, although Treg cells show no migration tendency into non-draining lymph nodes during tumor progression (Fig. 48). These results demonstrate Treg cell migration contributes to an influx in Treg cell populations during tumor progression, and their migration is dependent on L-selectin expression. Thus, L-selectin is required for Treg cell migration and accumulation, leading to a greater immunosuppressive environment during tumor progression.

Increases in Treg cell populations have been demonstrated in patients with cancer and results in poor prognosis (253, 258). However, the mechanisms responsible for influxes in Treg cells in tumor tissue and tumor-draining lymph nodes is not clearly understood. Here, we have demonstrated Treg cell migration may be responsible for elevated populations within these tissues. However, many studies have suggested that Treg cells may arise from conventional T cells within tumor microenvironments and within the tumor draining lymph node, resulting in iTreg cell populations (14, 268, 269). While these studies have focused specifically on nTreg migration during tumor progression, future studies on subset differences in iTreg cell adhesion molecule expression and migratory patterns may shed further light on Treg cell trafficking. In

addition, it is possible that migrated Treg cells may receive proliferative or apoptotic signals upon entry into tumors or tumor-draining lymph nodes. Thus, elucidating both proliferation and apoptosis of native and migrated Treg cells within these tissues will further our understanding of mechanisms controlling Treg cell populations during tumor progression.

Describing the role of L-selectin in mediating Treg cell migration has provided novel insight into homing of Treg cells to specific tissues during tumor progression. However, lymphocyte migration into lymph nodes is coordinated by a complex series of interactions that direct entry through HEV and inflamed endothelium. Specifically, chemokine receptors, such as CC chemokine receptor 7 (CCR7), directs lymphocyte homing into lymph nodes by binding to secondary lymphoid-tissue chemokine (SLC, Ref. 136). In addition, CCR4 expression by lymphocytes binds to its ligand CCL17, which is upregulated in venules during inflammation (175, 176). Therefore, determining Treg cell expression of chemokines may elucidate further mechanisms of Treg cell migration to tumors and tumor-draining lymph nodes. We have demonstrated cooperative interaction between L-selectin function and $\alpha_4\beta_7$ integrin interactions with VCAM-1. This interaction was required to enable efficient rolling and adhesion of Treg cells *in vitro*. However, whether L-selectin ligands and VCAM-1 are upregulated on vascular endothelium located in peri-tumoral tissue, tumors, or activated tumor-draining lymph node HEV has yet to be determined. While much research remains in describing other mechanisms involved in controlling Treg cell increases during tumor progression, these studies have demonstrated an L-selectin-dependent mechanism in which Treg cells specifically migrate to tumors and tumor-draining lymph nodes.

In conclusion, these studies provide evidence for L-selectin-dependent Treg cell migration to specific tissues during cancer. We have reported novel cooperative interactions between L-selectin function and $\alpha_4\beta_7$ integrin/VCAM-1 interactions *in vitro*. In addition, we have shown that Treg cells specifically increase in tumors and tumor-draining lymph nodes, and that L-selectin is required for optimal Treg cell distribution. Finally, we have demonstrated that Treg cells have a propensity to specifically migrate to tumors and tumor draining lymph nodes, and that their migration is dependent upon L-selectin. These studies are important for understanding immune suppression by Treg cells during cancer, and provide new insights into therapeutic strategies for chronic inflammation and immunotherapy.

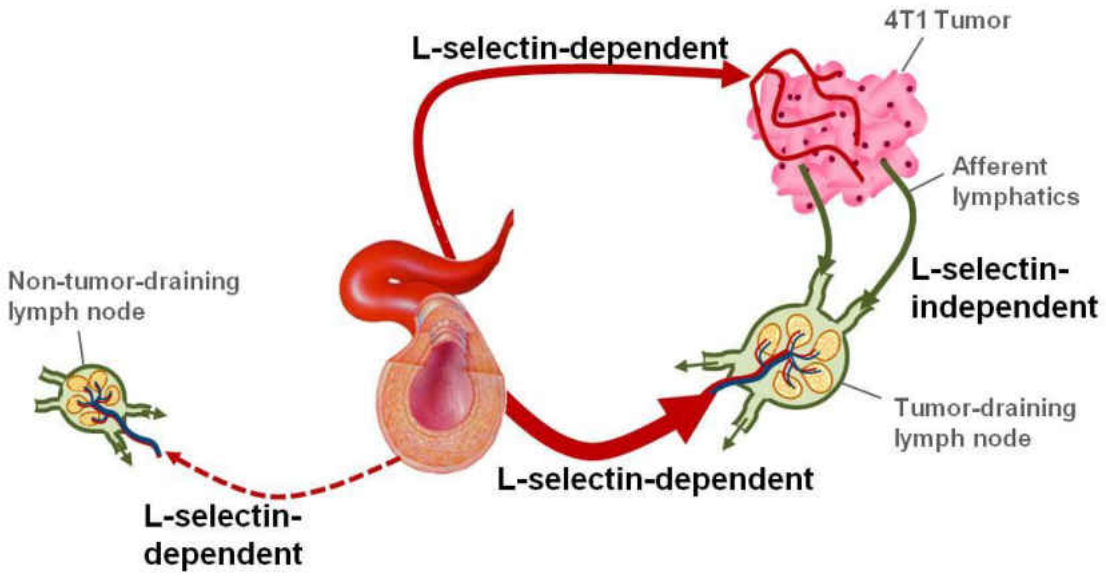


Figure 32. L-selectin is required for Treg cell migration into tumors and tumor-draining lymph nodes.

Regulatory T (Treg) cells migrate to tumors and tumor-draining lymph nodes during tumor progression in an L-selectin-dependent manner (solid arrows). The majority of Treg cell migration entry into tumor draining lymph nodes occurs from the blood (thick arrow). However, Treg cells are also able to enter tumor-draining lymph nodes through afferent lymphatics that drain the tumor, and is L-selectin independent (green arrows). While Treg cell migration to the non-tumor draining lymph nodes is also L-selectin dependent, there is no preferential migration during tumor progression (dashed arrow).

REFERENCES

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