Alteration of L-selectin by mAb, cytoskeletal association, and fatty acid membrane content
by Jeffrey Gordon Leid

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology
Montana State University
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Abstract:
L-selectin is an adhesion protein expressed on most leukocytes and is responsible for the initial event in the multistep process of transendothelial cell migration, tethering and rolling along the vessel wall. Leukocytes are captured from the blood stream by engagement of carbohydrate moieties, expressed on large molecular weight glycoproteins, by a calcium-dependent lectin domain expressed on L-selectin. L-selectin is expressed at the tips of the microvilli of leukocytes in humans and mice. Here, we extended this observation to bovine lymphocytes. Also, bovine &gama;δ T cells were smaller in size and contain greater than twofold the number of microvilli than αβ T cells. Engagement of L-selectin by its carbohydrate ligands may have functional consequences in the cell, such as calcium mobilization, tyrosine phosphorylation, and may also cause a conformational change in L-selectin that leads to stronger tethering and rolling interactions. L-selectin binding by an anti-L-selectin mAb, LAM1-116, caused a conformational change in L-selectin that was recognized by another anti-L-selectin mAb, EL-246. Both mAbs bind to distinct, functional epitopes on L-selectin. Furthermore, the conformational change induced in L-selectin by LAM1-116 predisposed L-selectin to cytoskeletal association, which was triggered by engagement of the EL-246 epitope by EL-246 mAb. Electron microscopy was employed to further characterize the nature of the lymphocyte cytoskeleton. The bovine lymphocyte cytoskeleton was dramatically different than an intact, resting cell. Compared to normal lymphocytes, mild detergent treatment of the lymphocyte generated a smaller, smooth-surfaced cell remnant that contained little cytoplasm and the cell nucleus. We also surveyed 28 human and bovine cell surface proteins and compared their patterns of cytoskeletal association to that of L-selectin. Our studies demonstrated that L-selectin fits a pattern of cytoskeletal association similar to that of other adhesion proteins involved in tethering and rolling. Finally, we have shown that enrichment of the lymphocyte cell membrane with polyunsaturated fatty acids inhibits the ability of L-selectin to associate with the cytoskeleton, and may reduce L-selectin-mediated adhesive events in vitro and in vivo.
ALTERATION OF L-SELECTIN BY MAB, CYTOSKELETAL ASSOCIATION, AND FATTY ACID MEMBRANE CONTENT

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology

MONTANA STATE UNIVERSITY
Bozeman, Montana

April, 2000
APPROVAL

of a dissertation submitted by

Jeffrey Gordon Leid

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date 26-April-2008
This dissertation is dedicated to my parents, Katherine M. Leid and R. Wes Leid, who have been both loving and wonderful inspirations to me throughout my life. Mom, I love you dearly and cherish every day I get to enjoy your company. Remember, you are the next in line! And Pops, what else can I say except that I have grown up just like you and of all the accomplishments in my life, I can think of none more fulfilling. I love you, thank you both.
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Abstract

L-selectin is an adhesion protein expressed on most leukocytes and is responsible for the initial event in the multistep process of transendothelial cell migration, tethering and rolling along the vessel wall. Leukocytes are captured from the blood stream by engagement of carbohydrate moieties, expressed on large molecular weight glycoproteins, by a calcium-dependent lectin domain expressed on L-selectin. L-selectin is expressed at the tips of the microvilli of leukocytes in humans and mice. Here, we extended this observation to bovine lymphocytes. Also, bovine γδ T cells were smaller in size and contain greater than twofold the number of microvilli than αβ T cells. Engagement of L-selectin by its carbohydrate ligands may have functional consequences in the cell, such as calcium mobilization, tyrosine phosphorylation, and may also cause a conformational change in L-selectin that leads to stronger tethering and rolling interactions. L-selectin binding by an anti-L-selectin mAb, LAM1-116, caused a conformational change in L-selectin that was recognized by another anti-L-selectin mAb, EL-246. Both mAbs bind to distinct, functional epitopes on L-selectin. Furthermore, the conformational change induced in L-selectin by LAM1-116 predisposed L-selectin to cytoskeletal association, which was triggered by engagement of the EL-246 epitope by EL-246 mAb. Electron microscopy was employed to further characterize the nature of the lymphocyte cytoskeleton. The bovine lymphocyte cytoskeleton was dramatically different than an intact, resting cell. Compared to normal lymphocytes, mild detergent treatment of the lymphocyte generated a smaller, smooth-surfaced cell remnant that contained little cytoplasm and the cell nucleus. We also surveyed 28 human and bovine cell surface proteins and compared their patterns of cytoskeletal association to that of L-selectin. Our studies demonstrated that L-selectin fits a pattern of cytoskeletal association similar to that of other adhesion proteins involved in tethering and rolling. Finally, we have shown that enrichment of the lymphocyte cell membrane with polyunsaturated fatty acids inhibits the ability of L-selectin to associate with the cytoskeleton, and may reduce L-selectin-mediated adhesive events in vitro and in vivo.
CHAPTER 1

INTRODUCTION

Leukocyte Trafficking

Historical Perspective

Cohnheim (1889) first described leukocyte rolling along venule walls almost a century before Gallatin and his colleagues (1983) suggested a function for L-selectin (1889). After his observation in 1889, others began to study the process of lymphocyte recirculation. At the turn of the 20th century, Davis and Carlson (1909), and later Sjovall (1936), hypothesized that lymphocytes exit the blood through the endothelium and enter the lymphatics. In 1972, Atherton and Born reported the adhesion of circulating polymorphonuclear leukocytes to blood vessel walls (1972). Exquisite studies by James Gowans and others in the late 1950s and early 1960s led to the foundation of what we now understand to be a multistep, highly regulated process of lymphocyte recirculation from the blood, into the lymph, and back again into the blood. Specifically, Gowans reported on the effects of continuous re-infusion of lymphocytes on thoracic duct lymphocyte output in a rat model (1957). Two years later, he reported on the recirculation of lymphocytes from the blood to the lymph in the rat (Gowans, 1959). Further studies conducted by McGregor and Gowans demonstrated that if the recirculating pool of lymphocytes is eliminated, a primary immune response against...
sheep red blood cells or skin grafts does not develop (1963). Reconstituting the animal with competent lymphocytes, however, results in restoration of the immune response. Thus, not only is the presence of lymphocytes important for immune system function, but the trafficking of lymphocytes is also vital to the generation of an immune response.

**Constitutive Lymphocyte Homing**

Naive lymphocytes traffic to secondary lymphoid sites in the body (Butcher et al., 1999). This type of homing is called constitutive homing, or lymphocyte recirculation, because it occurs continuously and in the absence of inflammation. Naive lymphocytes enter secondary lymphoid organs, such as peripheral lymph nodes (PLNs), Peyer's patches (PPs), and tonsils, and if they do not encounter foreign antigen, exit through the efferent lymphatics, reenter the blood stream, and traffic to another secondary site in the body (Mackay et al., 1990). Constitutive homing allows lymphocytes to continually recirculate through secondary lymphoid organs, where foreign antigens are presented, and enhances the chance that antigen-specific B or T cells recognize these antigens and respond accordingly (Butcher et al., 1999).

**Tissue-selective Trafficking**

Once a naive cell encounters antigen and proliferates, it becomes an effector/memory cell. Memory cells exhibit a different homing pattern than naive cells and often recirculate back to the place they originally encountered antigen (Mackay et al., 1992, Bradley and Watson, 1996, and Butcher et al., 1999). In general, memory cells
home preferentially to peripheral tissues, such as the skin, as well as other areas, such as
the gut mucosa (Mackay, 1992). Moreover, CD4+ memory T cells do not migrate into
PLN in the absence of antigen (Bradley et al., 1999). Lymphocytes isolated from the
efferent lymph of sheep mesenteric lymph nodes (MLN) preferentially home to sheep
mucosal tissues upon reinjection into that animal. Similarly, lymphocytes isolated from
the efferent lymph of peripheral lymph nodes migrate preferentially back to the PLN
(Reynolds et al., 1982, Scollay et al., 1976, and Cahill et al., 1977). In addition, sheep
lymphocytes collected from gut-draining lymphatics, having been exposed to foreign
antigens, labeled with FITC, and injected into syngeneic animals, home to the gut versus
the peripheral lymphatics (Mackay et al., 1992). The cells were re-isolated, phenotyped,
and found to consist primarily of memory lymphocytes (Mackay et al., 1992). Thus,
these studies, and others, suggest that lymphocytes traffic in a preferential manner to
specific sites in the body and their patterns of trafficking can be influenced by the tissue
of origin. A variety of tissues/ organs are involved in tissue-specific homing, as illustrated
in Table 1.1 below.

Table 1.1. Tissues Involved in Tissue-specific Homing

<table>
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<th>Tissue Sites</th>
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<td>1) PLN</td>
<td>(Reynolds et al., 1982, and Butcher, 1986)</td>
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<tr>
<td>2) Gut-associated lymphoid tissue</td>
<td>(Cahill et al., 1977, and Butcher, 1999)</td>
</tr>
<tr>
<td>3) Lung-associated lymphoid tissue</td>
<td>(Butcher, 1986 and Yednock and Rosen, 1989)</td>
</tr>
<tr>
<td>4) Nasal-associated lymphoid tissue</td>
<td>(Csensits et al., 1999)</td>
</tr>
<tr>
<td>5) Skin</td>
<td>(Picker et al., 1993)</td>
</tr>
<tr>
<td>6) Gut lamina propia</td>
<td>(Rose et al., 1978, and Butcher, 1999)</td>
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Lymphocyte Subset-specific Homing

Besides the naive and memory subsets of lymphocytes described above, other lymphocyte subsets exist that exhibit specific homing properties. Gamma delta T cells, a recently defined subset of T cells, preferentially home to extralymphoid, epithelial sites in the body, such as skin and mucosal tissues, and homing correlates with specific TCR usage (Cahill et al., 1996). For example, high numbers of Vγ4/Vδ1 γδ T cells are found in the reproductive tract of mice (Mincheva-Nilsson et al., 1997, and Rakasz et al., 1998). Bovine γδ T cells also predominate in the spleen (Holder et al., 1999, and Wilson et al., 1999). Thus, a variety of lymphocyte subsets exist that exhibit specific homing characteristics.

Inflammation-induced Homing

In addition to the homing patterns described above, there exists a second general type of tissue-specific homing mediated by inflammation. Inflammation-induced trafficking is a response to insult and injury in the animal and the process preferentially recruits cells with specific effector functions. Neutrophils, leukocytes that normally do not home to any tissues, are recruited to sites of inflammation (Jutila et al., 1989a). Another type of leukocyte, called a monocyte, is also recruited from the blood to tissue sites of inflammation (Jutila et al., 1989b, and Spertini et al., 1992). Natural killer (NK) lymphocytes, so-called because they do not need previous exposure to antigen to function and are effective anti-tumor killing cells, also show a preference for mucosal
tissue in response to IL-2 activation (Uksila et al., 1997). In addition, lymphocytes isolated from sites of inflammation migrate back to the site where the inflammatory event originally occurred, and also home more effectively to other sites of tissue inflammation (Chin and Hay, 1980, Issekutz and Hay, 1980, Picker and Butcher, 1992). Therefore, not only are lymphocytes recruited to inflammatory sites, but other leukocytes are as well, including granulocytes, monocytes, and NK cells (Butcher et al., 1999).

**Leukocyte-endothelial Cell Interactions**

In order for leukocytes in the blood to traffic to a specific site in the body, they must first interact with the endothelium. Constitutive homing interactions primarily take place in postcapillary venules (PCV) of the various secondary lymphoid organs on specialized endothelial cells, termed high endothelial venules (HEV) (Stamper and Woodruff, 1976, Picker and Butcher, 1992, Dianzani and Malavasi, 1995, Stoolman et al., 1984, and Stoolman, 1989). HEV are plump, cuboidal endothelial cells that, because of their large surface area, are well-suited for lymphocyte binding. The regions of postcapillary venules expressing HEV look physiologically different than the regular, flattened appearance of the rest of the endothelial cells lining the PCV. In humans and mice, each of the secondary lymphoid organs, with the exception of the spleen, have areas on the endothelium where HEV are present. Memory cells, like naive cells, also preferentially traffic through PCV, into the tissues where they first encountered antigen (Mackay et al., 1992).
As one can imagine from the above descriptions of homing, leukocyte-endothelial interactions exhibit substantial specificity. Mouse lymphocytes incubated on thin frozen sections of lymph nodes and PP, as described originally by Stamper and Woodruff, specifically adhere to HEV (1976, and Rosen et al., 1985). Rodent and human lymphocytes also display similar characteristics (Stoolman et al., 1987). Monocyte adhesion is also mediated by HEV binding (Grober et al., 1993). However, monocyte attachment to the endothelium also occurs in the absence of HEV (Grober et al., 1993). Collectively, the process of leukocyte trafficking from the blood into secondary lymphoid or other tissues, is a highly regulated and specific process, mediated in part by leukocyte-endothelial interactions.

Multistep Model of Leukocyte Trafficking

Leukocytes travel through the blood stream at ~4000 μm/sec (Bargatze et al., 1995). In order for tight adhesion to take place, the leukocyte must slow down. The adhesion proteins expressed on leukocytes and the endothelium are the molecular braking mechanisms, slowing the cells down 100-fold to ~40 μm/sec (Bargatze et al., 1995, and Figure 1.1A). This first step in the multistep process is called tethering and rolling. Tethering is the formation of labile adhesion on the vessel walls which allows for the subsequent rolling of leukocytes in the direction of blood flow (Fabbri et al., 1999). The second event occurs with activation of the leukocyte leading to tight adhesion (Butcher et al., 1999). Through G-protein-linked signaling receptors, the leukocyte undergoes
functional alterations, adheres tightly and flattens on the endothelium, usually within 20 seconds (Bargatze and Butcher, 1993, and Butcher et al., 1999, Figure 1.1B and C). If further G-protein-mediated signals are present, the leukocytes eventually transmigrate through the endothelium (Butcher et al., 1999, and Figure 1.1D). Importantly, each step is a reversible process and if specific stimuli do not exist, the cells release back into the blood stream.

Figure 1.1. The multistep model of leukocyte transmigration showing rolling (A), activation (B) and tight adhesion (C), and transendothelial migration (D).
Adhesion Molecules Involved in Rolling

A variety of adhesion molecules are involved in rolling as described in the previous model (Figure 1.1). In addition, a number of receptor/ligand pairs contribute to the distinct tissue-selective homing patterns. Table 1.2 lists some of the adhesion molecules which have been shown to be involved in rolling.

Table 1.2. Receptor/ligand Pairs Involved in Rolling

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<tr>
<th>Homing Adhesion Receptor</th>
<th>Vascular Addressin</th>
<th>Tissue of Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β7</td>
<td>MAdCAM-1</td>
<td>GALT</td>
</tr>
<tr>
<td>α4β1</td>
<td>VCAM-1</td>
<td>Inflammation</td>
</tr>
<tr>
<td>CLA</td>
<td>E-selectin</td>
<td>Skin</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin</td>
<td>Acute Inflammation</td>
</tr>
<tr>
<td>L-selectin</td>
<td>PNAd</td>
<td>PLN</td>
</tr>
</tbody>
</table>

Three major families of adhesion molecules exist, the selectins, the integrins, and the immunoglobulin-like superfamily. The selectin family of adhesion proteins mediates the initial interaction of the leukocyte with the endothelium, an interaction which, as mentioned above, is termed rolling. Integrins mediate the tight adherence of leukocytes to the endothelium as well as participate in the process of transmigration. The immunoglobulin superfamily of adhesion proteins are expressed on the endothelium and act as counterreceptors for the integrins. As my dissertation focuses on L-selectin, the remaining portion of this discussion will provide a detailed overview of this adhesion protein.
L-selectin

History of L-selectin

Since the early 1960’s, lymphocytes have been known to associate with specialized vessels, termed postcapillary high endothelial venules. Indeed, in 1964, James Gowans and E.J. Knight (1964) noted this interaction and postulated potential homing mechanisms. However, it was not until 1983, that scientists began to understand the molecular process of homing in more detail with the discovery of L-selectin (Gallatin et al., 1983). In their study, Gallatin and colleagues blocked lymphocyte binding to HEV in the Stamper and Woodruff *ex vivo* adhesion assay with a monoclonal antibody, MEL-14 (1983, and Stamper and Woodruff, 1976). Additionally, injection of MEL-14 into recipient animals blocked lymphocyte homing to PLN *in vivo*. These studies led to the original characterization of L-selectin as the PLN homing receptor.

As further research was performed, it became clear that the peripheral homing receptor, which was recognized by the MEL-14 mAb, interacted with the endothelium through a carbohydrate binding activity. In 1984, Stoolman and colleagues demonstrated that phosphomannosyl carbohydrates, such as phosphomannan (PPME), inhibited lymphocyte binding to HEV (Stoolman et al., 1984). Similarly, PPME-coated beads bind to lymphocytes in a calcium-dependent manner, and this interaction is blocked by MEL-14 mAb treatment (Yednock et al., 1987a and b). Further studies corroborated the lectin-like activity of the MEL-14 antigen (Geoffrey and Rosen, 1989, and Imai et al., 1990).
Together, these data suggested that the peripheral homing receptor contained a carbohydrate binding domain that is responsible for the interaction between lymphocytes and endothelium.

Extensive cloning work done in the late 1980s led to the molecular characterization of L-selectin. In 1989, five different papers were published describing the cloning of cDNA encoding L-selectin in the mouse and human (Lasky et al., 1989, Siegelman et al., 1989, Siegelman and Weissman, 1989, Bowen et al., 1989, and Tedder et al., 1989) and subsequent work identified this protein in the cow (Walcheck et al., 1992). The cloning work confirmed the functional studies mentioned above and established that L-selectin contains a lectin domain that binds carbohydrate. During this same time period, two other adhesion proteins were cloned, E-selectin and P-selectin, which shared homology to L-selectin (Bevilacqua et al., 1989, and Johnston et al., 1989). E-selectin is an inducible adhesion molecule that is present on endothelial cells and is an active participant in the process of leukocyte rolling (Bevilacqua et al., 1987, and Jutila et al., 1994). P-selectin, also an inducible adhesion molecule with similar functions to L- and E-selectin, is found in alpha granules of platelets and Weible-Palade bodies in endothelial cells (Bevilacqua et al., 1989, Jutila et al., 1994, and McEver et al., 1989). In 1991, scientists agreed upon a common name, "selectin," for these three newly described cell adhesion molecules (Bevilacqua et al., 1991).
Structure of L-selectin

All three selectins contain five different domains: a Ca\textsuperscript{2+}-dependent C-type lectin domain; an epidermal growth factor-like domain (EGF); short consensus repeat domains (SCR) that have homology to complement binding proteins; a transmembrane domain, and a small cytoplasmic tail. L-selectin differs from E- and P-selectin in the number of SCR domains. In the mouse, L-selectin has two SCRs, whereas E-selectin has five and P-selectin has eight. In humans, E-selectin has six SCR domains and P-selectin has nine. The number of SCR’s in L-selectin does not vary from species to species.

![Diagram of the selectin family](image)

Figure 1.2. Diagram of the selectin family of adhesion molecules showing their common domains; Lectin, EGF, SCR, Transmembrane, and Cytoplasmic tail domains.
The C-type lectin domain serves as the calcium-dependent carbohydrate binding domain for all three selectins (Stoolman et al., 1984, and reviewed in Varki, 1994, Jutila 1996, and Kansas, 1996). Treatment of any of the selectins with EDTA, a divalent chelator of calcium, diminishes the ability of each to bind carbohydrates (Yednock et al., 1987b). The EGF and SCR domains are important to the structure of the molecule, although some mAbs directed against these domains block the function of L-selectin under flow, suggesting that these domains also contribute to ligand binding (Siegelman et al., 1990, Watson et al., 1991, and Kansas et al., 1991). The cytoplasmic tail has also been shown to be important, especially in L-selectin function (Kansas et al., 1993, Evans et al., 1999, and Kahn et al., 1998). Of all the domains, homology is highest between the lectin domains of the selectins, which accounts for their ability to recognize overlapping ligands (Mebius and Watson, 1993, Tu et al., 1999, and Kansas, 1996).

Expression of L-selectin

Besides its expression on lymphocytes, L-selectin is also found on other leukocytes including monocytes, eosinophils, and neutrophils (Lewinsohn et al., 1987, and Jutila et al., 1989b). As with the studies on lymphocytes, bone marrow neutrophils bind to HEV in frozen sections and their binding is blocked by MEL-14 (Lewinsohn et al., 1987, and Jutila et al., 1989a). Recently, L-selectin expression has also been reported on dendritic cells, and may account for the ability of dendritic cells to home to PLN in vivo (Munro et al., 1996, and Vremec and Shortman, 1997).
Different cells express varying amounts of L-selectin. Walcheck and colleagues (1994) have shown that bovine γδ T cells express 2-5 times the amount of L-selectin than do αβ T cells. At the time, it was not clear whether this was due simply to enhanced L-selectin syndissertation or a physical difference in γδ versus αβ T cells. The functional nature of varying levels of L-selectin on αβ versus γδ T cells has not been well defined, but our lab has shown that γδ T cells exhibit highly efficient rolling interactions by in vitro assays done under physiological shear (Jutila et al., 1994, and Jutila and Kurk, 1996).

L-selectin is located at the tips of the microvilli of leukocytes (Picker et al., 1991, and Bruehl et al., 1996). Other adhesion proteins involved in rolling also are expressed at the tips of leukocyte microvilli including E-selectin ligand-1 and P-selectin glycoprotein ligand-1 (Steegmaier et al., 1997, and Moore et al., 1995). Thus, these adhesion proteins involved in the first step of the multistep pathway are well positioned for their role in leukocyte/endothelial rolling interactions.

**L-selectin Ligands**

A variety of ligands have been defined for L-selectin (Table 1.3).
Table 1.3. L-selectin Ligands

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Expression</th>
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<tbody>
<tr>
<td>Peripheral Node Addressin</td>
<td>Endothelium</td>
</tr>
<tr>
<td>Mucosal Addressin Cell Adhesion Molecule 1</td>
<td>Endothelium</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Endothelium</td>
</tr>
<tr>
<td>P-selectin Glycoprotein Ligand 1</td>
<td>Leukocyte</td>
</tr>
</tbody>
</table>

Peripheral node addressin (PNAd) was originally defined as a group of 5 different molecular weight proteins that are precipitated by the MECA-79 mAb (Streeter et al., 1988). Four of these have been well characterized and include Glycam-1, CD34, Sgp-200, and a recently defined protein called podocalyxin (Lasky et al., 1992, Baumhueter et al., 1993, Berg et al., 1991, Hemmerich et al., 1994, and Sassetti et al., 1998). Most are present on HEV and can mediate rolling interactions with L-selectin. However, Glycam-1 is a soluble protein and CD34 knockout mice do not show a decrease in the rolling and homing capacities of lymphocytes (Lasky et al., 1992, and Suzuki et al., 1996). Thus, these two carbohydrate ligands may not play a vital role in L-selectin-mediated constitutive homing. Podocalyxin is currently under investigation and has not yet been shown to be important in vivo (Sassetti et al., 1998). Sgp-200 can be present in both membrane-bound and soluble forms and can mediate L-selectin binding (Hemmerich et al., 1994). However, its interactions with L-selectin are still under investigation and the molecule has yet to be cloned. Therefore, it is not clear whether one or all of the MECA-79 antigens are the principal L-selectin ligands on HEV. Together though, these antigens are important in L-selectin-mediated constitutive homing as MECA-79 blocks homing of
lymphocytes \textit{in vivo} and blocks HEV binding by lymphocytes \textit{in vitro} (Streeter et al., 1988).

Mucosal addressin cell adhesion molecule-1, or MAdCAM-1, is the vascular addressin that was originally defined to bind \(\alpha4\beta7\), but also binds L-selectin (Briskin et al., 1993, and Berg et al., 1993). It is a 58-66 kD glycoprotein that has the unique feature of containing both immunoglobulin-like domains as well as mucin-like domains in its structure (Briskin et al., 1993). Mucins are glycoproteins rich in serine and threonine residues and tend to be heavily O-glycosylated at these sites. This point is important as most of the defined L-selectin ligands are mucins that contain clustered serine/threonine residues which serve as linkage points to the carbohydrate moieties that L-selectin recognizes (Varki, 1994, and Kansas, 1996). MAdCAM-1 is expressed in PP, MLN, and lamina propia, and MECA-367, a mAb that specifically binds to MAdCAM-1, blocks lymphocyte recognition of intestinal postcapillary venules \textit{in vitro} and \textit{in vivo} (Streeter et al., 1988 and Nakache et al., 1989). L-selectin binds to MAdCAM-1 when the PNAd carbohydrates are expressed on its surface (Berg et al., 1993) and through this interaction, L-selectin plays a role in homing to mucosal sites as well as peripheral sites. Rolling of lymphocytes on PPs is initiated by the L-selectin/MAdCAM-1 interaction but is sustained by the binding and subsequent rolling of \(\alpha4\beta7\) on MAdCAM-1 (Bargatze et al., 1995).
Picker and colleagues originally reported that neutrophil L-selectin presents carbohydrates to E- and P-selectin and suggested that selectin-selectin binding could mediate rolling interactions (Picker et al., 1991). Kishimoto and colleagues expanded on this and reported that L-selectin and E-selectin mediate common adhesion pathways in vitro (Kishimoto et al., 1991). Other studies have implicated L-selectin-E-selectin interactions as well (Zoller et al., 1997, and Jones et al., 1997). Zoller and colleagues (1997) demonstrated that human neutrophil L-selectin, but not mouse, binds to E-selectin in vitro. Jones and colleagues (1997) confirmed these observations by demonstrating that an E-selectin chimera expressing the E-selectin extracellular domain fused to an immunoglobulin G constant region, specifically precipitated L-selectin from neutrophil membrane lysates. Thus, it is clear that E-selectin can be a ligand for L-selectin, depending upon the type of leukocyte expressing L-selectin. However, this interaction has yet to be shown to be important in vivo.

In addition to mediating leukocyte-endothelial interactions, L-selectin also mediates leukocyte-leukocyte interactions (Bargatze et al., 1994, and Jutila and Kurk, 1996). One counterreceptor expressed on the leukocyte cell surface that binds to L-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) (Guyer et al., 1996, Spertini et al., 1996, Tu et al., 1996, and Walcheck et al., 1996). PSGL-1 is a 230 kD homodimer expressed on most leukocytes and was originally defined as the primary ligand for P-selectin (Moore et al., 1992, and Sako et al., 1993). MAbs against PSGL-1 that block P-selectin-mediated rolling interactions do not block L-selectin-mediated interactions to the
same extent, suggesting the presence of another important ligand in L-selectin-mediated leukocyte-leukocyte interactions (Walcheck et al., 1996). Further characterization found that E-selectin can also bind PSGL-1, yet the importance of these interactions are not clear (Asa et al., 1995, Goetz et al., 1997, and Borges et al., 1997). PSGL-1 knockout mice show a dramatic reduction in P-selectin-mediated lymphocyte rolling and capture whereas PSGL-1 is not needed for E-selectin mediated rolling events in vivo (Yang et al., 1999). Future investigation may clarify the issue as other ligands for L- and E-selectin are discovered.

**L-selectin, Adhesion, and Rolling**

As mentioned earlier, Gallatin and colleagues (1983) first demonstrated a direct interaction between the lymphocyte and endothelium using the mAb, MEL-14. Lewinsohn and Jutila (1987 and 1989b, respectively) broadened this observation to L-selectin on other leukocytes. Lewinsohn (1987) provided the first evidence of a common molecular mechanism shared by all leukocytes in their recognition of host endothelium. Jutila (1989b), by MEL-14 mAb binding to L-selectin, ascertained that pretreated neutrophils do not home to inflamed tissues and neutrophils deficient in L-selectin lacked trafficking capabilities. With new intravital microscopy techniques, scientists began to biochemically characterize leukocyte rolling interactions on endothelium in the animal. von Andrian demonstrated that neutrophils use L-selectin to roll on activated endothelium in rabbits (1991). Ley and colleagues confirmed these results showing that L-selectin
mediates leukocyte rolling in mesenteric venules in rats in vivo (1991). Recently, L-selectin was shown to be important in HEV-mediated rolling in vivo, substantiating the early ex vivo HEV binding assays (von Andrian, 1996).

L-selectin also mediates rolling interactions on other immobilized leukocytes (Bargatze et al., 1994). Bargatze and colleagues, using an in vitro closed loop circulation assay, showed that neutrophils roll on other adherent neutrophils under shear forces similar to those found physiologically, and that rolling is dependent upon the presence of L-selectin on the rolling cells, as determined by mAb blocking studies (1994). Gamma delta T cells, a subset of lymphocytes found in particularly high numbers in ruminants, also exhibit L-selectin-dependent rolling interactions on other immobilized γδ T cells (Jutila and Kurk, 1996). It has been proposed that leukocyte-leukocyte rolling interactions serve to augment leukocyte-endothelial interactions (Bargatze et al., 1994, and Alon et al., 1996).

L-selectin Regulation

Neutrophils isolated from sites of inflammation, which no longer have the capacity to home to other tissues, lack L-selectin, suggesting a regulatory role for L-selectin cleavage (Jutila et al., 1989a). Following activation of either lymphocytes or myeloid cells with chemotactic factors or phorbol esters, there is a rapid increase in functional avidity which, under most circumstances, is followed by shedding of the cell surface molecule (Spertini et al., 1991, Kishimoto, et al., 1989, Griffin, et al., 1990 and
Smith, et al., 1991). There is an inverse relationship between the downregulation of L-selectin and the activation of the β2 integrin, Mac-1 (Jutila et al., 1989a, and Kishimoto et al., 1989). L-selectin is shed from the cell surface of neutrophils while Mac-1 is upregulated (Jutila et al., 1989a, and Kishimoto et al., 1989). The basis for the increased functional avidity in L-selectin immediately following activation of the leukocyte is poorly understood, but it may be due to phosphorylation of L-selectin, clustering of the protein, cytoskeletal association of L-selectin through its cytosplasmic tail, or conformational changes induced in L-selectin (Haribabu, et al., 1997, Li et al., 1998, and Evans et al., 1999). Hyperthermic treatment of lymphocytes also increases L-selectin function in vitro and in vivo (Wang et al. 1998). In their study, Wang and colleagues demonstrated that hyperthermic-treated lymphocytes bind better to HEV in ex vivo assays and home more efficiently to PLN in vivo (1998).

L-selectin shedding is caused by proteolysis, which can be blocked by protease inhibitors that target a unique membrane-bound metalloproteinase (Kishimoto et al., 1989, Jutila et al., 1991, Kishimoto et al., 1995, Feehan et al., 1996, Walcheck et al., 1996, and Bennet et al., 1996). Endoproteolytic release of L-selectin from the surface of leukocytes is regulated by certain structural requirements of L-selectin and mutational analysis of the cleavage site demonstrates that conformation is the most important aspect in L-selectin shedding (Chen et al., 1995, and Migaki et al., 1995). A recent report by Kahn and colleagues suggests calmodulin, a calcium regulatory protein that specifically co-
precipitates with L-selectin through a direct association with the cytoplasmic tail, also regulates L-selectin shedding (1998). Calmodulin inhibitors disrupt L-selectin-dependent adhesion by inducing proteolytic release of L-selectin from the cell surface (Kahn et al, 1998).

The putative metalloprotease that cleaves L-selectin has been recently defined. Tumor necrosis factor alpha converting enzyme (TACE), an enzyme responsible for cleaving TNF-α, has been implicated in L-selectin cleavage from the cell surface (Peschon et al., 1998). Peschon and colleagues generated mice deficient in the active form of TACE by mutating the Zn²⁺ binding domain, thus inactivating its metalloproteinase activity (1998). However, this mutation is lethal so embryonic cells were used to characterize TACE activity on L-selectin. Embryonic thymocytes isolated from the TACE mutants did not downregulate L-selectin from the cell surface when treated with PMA in comparison to wild type embryonic thymocytes (Peschon et al., 1998). Also, TACE cleaves a peptide that matches the proteolytic site on L-selectin (Peschon et al., 1998). Hence, TACE mediates L-selectin cleavage from the cell surface.

L-selectin shedding may allow leukocytes to break their tight bonds with native ligands and proceed with tight adhesion and transmigration (Kishimoto et al., 1991). A recent report suggests that in vivo, a hydroxamic acid-based metalloproteinase inhibitor (KD-IX-73-4) that blocks L-selectin cleavage, actually slows the rolling velocity of leukocytes compared to untreated cells (Hafezi and Ley, 1999). Another important
measure of L-selectin function is the number of rolling cells per unit time of observation (rolling flux) and KD-IX-73-4 treatment increases the rolling flux of leukocytes observed \textit{in vivo} (Hafezi and Ley, 1999). Additionally, the number of firmly adhering leukocytes, as well as the number of transmigrating cells, increased (Hafezi and Ley, 1999). These data suggest that L-selectin shedding significantly impacts leukocyte recruitment and provides a physiological role for L-selectin shedding \textit{in vivo}, expanding on Jutila and colleagues' original observation that leukocytes isolated from inflammatory sites lack L-selectin (Jutila et al., 1989a).

**L-selectin Signaling**

L-selectin has been implicated in cellular signaling events which regulate the function of other adhesion molecules on the cell surface and the activation state of the leukocyte. Engagement of L-selectin on neutrophils by mAb leads to activation and tight adhesion, potentiates the oxidative burst, and enhances tyrosine phosphorylation as well as activating MAP kinase (Waddell et al., 1994, Crocket-Torabi et al., 1995, Simon et al., 1995, and Waddell et al., 1995). In these studies, crosslinking of L-selectin by a secondary reagent specific to the anti-L-selectin mAb, or by ligand binding (Glycam-1), is requisite for the signaling events. Other examples exist where engagement of L-selectin by primary antibody alone, directly activates the cell. Binding of L-selectin by a mAb directed against a highly conserved region of L-selectin alone, causes activation of neutrophils and their subsequent tight adherence (Steeber et al., 1996). Engagement of
neutrophil L-selectin by sulfatides, carbohydrate moieties, increases cytosolic free Ca\(^{+2}\), increases expression of TNF-\(\alpha\) and IL-8 mRNA, and also transiently induces oxygen radicals (Laudanna et al., 1994, and Bengtsson et al., 1996). More recently it has been reported that neutrophils arrest on ICAM-1 when activated independently through L-selectin under physiological shear force (Gopalan et al., 1997). In addition, human neutrophil L-selectin signaling causes alterations in the cytoskeleton as well as colocalization with CD18 (Simon et al., 1999). This observation, and those above, demonstrate a correlation between L-selectin engagement, albeit some with artificial ligands, and downstream activation events.

L-selectin and the Cytoskeleton

The actin-based cytoskeleton is made up of three primary components, microtubules, microfilaments, and intermediate filaments, and is vital in maintaining the integrity of various immune cells in the body, including endothelial cells and leukocytes (Kim et al., 1989, and Wechezak et al., 1989, and reviewed in Hall, 1998). Recently, much work has focused on the actin cytoskeleton and lymphocyte function (Penninger and Crabtree, 1999). Numerous studies have shown that the functional capacity of lymphocytes, as well as proteins contained internally and externally, depends on a competent actin cytoskeleton. Cytoskeletal defects are important in various disease states including cancer (Saunders et al., 2000), Wiskott-Aldrich Syndrome (Gallego et al., 1997, Wu et al., 1998, Zicha et al., 1998, Snapper and Rosen, 1999, and Trasher and
Burns, 1999), B-cell function (Cheng et al., 1999), HIV pathogenesis (Pearce-Pratt et al., 1994, Soll and Kennedy, 1994, Soll, 1997, Fackler et al., 1999, and Wang et al., 2000) and other immune cell function deficiencies (Zhang et al., 1999, and Roberts et al., 1999). Thus, the ability of a protein to associate with the cytoskeleton plays a vital role in its function.

Cytoskeletal association of adhesion molecules has been investigated for some time. Most studies have focused on integrin function as it relates to the cytoskeleton controlling the avidity of β1 and β2 integrins in leukocytes (Shaw et al., 1990, Pavalko and LaRoche, 1993, Otey et al., 1993, Pardi et al., 1992, Pavalko and Otey, 1994, and Sampath, et al., 1998). These reports suggest that integrins associate with detergent-resistant membranes (DRMs) through direct binding of the integrin cytoplasmic tail to the actin cytoskeleton. A variety of other proteins have been studied as well, including CD2, CD4, CD8, CD44, CD45, and class I MHC (Geppert and Lipsky, 1991, and Gur et al., 1997). Depending upon the treatment of proteins by either primary antibody alone or crosslinking, they can be induced to associate with the cytoskeleton (Geppert and Lipsky, 1991, and Evans et al., 1999). However, some molecules are linked directly to the cytoskeleton immediately after syndissertation and transport from the Golgi (Brown and Rose, 1992, Varma and Mayor, 1998, and Melkonian et al., 1999).

Numerous signaling molecules constitutively associate with the cytoskeleton through GPI-anchored interactions that form what some refer to as “lipid rafts”, which
play a role in T cell activation (Moran and Miceli, 1998). The concept of lipid raft was first proposed by Brown and Rose (1992) and has since received much attention. In addition to lipid rafts, supramolecular activation clusters (SMAC) exist as organized contact sites at the physical interface between T cells and antigen presenting cells and the proteins involved at such sites are linked to the cytoskeleton (Monks et al., 1997, Shaw and Dustin, 1997, and Monks et al., 1998). Recent studies have also elucidated the importance of the Ras superfamily of small guanosine triphosphates (GTPases), especially the Rho family, and the actin cytoskeleton in immune cell function (Hall, 1998, and Magee and Marshall, 1999). Indeed, Rho family deficiencies, in which cytoskeletal interactions are affected, show abnormalities in neutrophil function and host defense, decreases in integrin-mediated cell adhesion, decreases in cell motility in neutrophils, and abnormalities in growth factor induced chemotaxis in neutrophils and macrophages (Roberts et al., 1999, Laudanna et al., 1996, Stasia et al., 1991, Allen et al., 1997, Allen et al., 1998, and Zicha et al., 1998). Rho GTPases also induce T cell adhesion to MAdCAM-1, and control migration and polarization of adhesion molecules and ERM components in T lymphocytes, and defects in these GTPases affect T cell trafficking (Zhang, et al., 1999, and del Pozo et al., 1999).

Linkage to the cytoskeleton is also important to the function of L-selectin. The cytoplasmic tail of L-selectin is required for leukocyte rolling and adhesion (Kansas et al, 1993). Deletion mutants lacking the carboxyl terminal 11 amino acids of the cytoplasmic tail of L-selectin do not bind to HEV nor establish rolling interactions in vivo (Kansas et
The cytoplasmic tail of L-selectin binds to the cytoskeletal-associated proteins α-actinin and vinculin (Pavalko et al., 1995). Evans and colleagues determined by antibody crosslinking, hyperthermic treatment, and ligand binding assays, that the cytoplasmic tail of L-selectin is important to L-selectin function by regulating linkage to the actin cytoskeleton through specific binding interaction (1999). Presumably, this interaction is mediated by α-actinin binding to actin and the cytoplasmic tail of L-selectin. Specific concentrations of cytochalasin B, which inhibit actin polymerization, block L-selectin-mediated rolling, although it is not clear what mechanisms are involved in the inhibition of rolling interactions (Kansas et al., 1993). These data suggest a causal relationship between the ability of L-selectin to associate with the cytoskeleton and its ability to form strong tethers and thus high avidity rolling interactions with its carbohydrate ligands. However, the study by Evans and colleagues does not directly address this question.

Summary

In this dissertation, I have explored the nature of L-selectin regulation by a variety of techniques in an effort to test the following hypothesis:

Expression and conformation of L-selectin, as well as fatty acid composition of the cell membrane, impact its linkage to the cytoskeleton.
In regards to this hypothesis, the following issues are addressed.

1) Ultrastructure of L-selectin expression on bovine lymphocytes.

2) Characterization of a mAb-induced conformational change in L-selectin and impact of this conformational change on L-selectin cytoskeletal linkage.

3) Comparison of L-selectin linkage to the cytoskeleton with the linkage of other important cell surface molecules.

4) Alteration of the linkage of L-selectin to the cytoskeleton.

Brief Overview of Chapter Contents

The second chapter deals with the ultrastructure and expression of L-selectin on $\alpha\beta$ versus $\gamma\delta$ T cells and was published in the Journal of Leukocyte Biology as detailed below:


Chapter 3 focuses on a conformational change in L-selectin that predisposes the protein to cytoskeletal association and some of the work was done in collaboration with Drs. Thomas Tedder and Douglas Steeber in the Department of Immunology at Duke University Medical Center. Chapter 4 reports on the comparison of the cytoskeletal association of L-selectin to that of other functionally characterized proteins as well as characterizes the role of PUFA treatment on L-selectin cytoskeletal linkage. Chapter 5 is
a summary of the work presented in chapters 2-4 as well as other collaborations I have worked on during my graduate program.
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CHAPTER 2

COMPARATIVE ULTRASTRUCTURE AND EXPRESSION OF L-SELECTIN ON BOVINE αβ AND γδ T CELLS

Introduction

Although γδ T cells were discovered over a decade ago, their functions are still not clearly defined. Some suggest that these cells play a role in the initial host response against a number of different bacterial, viral, and parasitic agents, contributing to what is referred to as “the first line of defense” (Libero, 1997). Gamma delta T cells have many characteristics that distinguish them from other T cells, including molecularly unique receptors for antigens (γδ TCRs) (Raulet, 1989, and Hein and Mackay, 1991), lack of either CD4 or CD8 expression (Mackay and Hein, 1989) though some γδ T cells express a low level of CD8, and unique trafficking behaviors in the animal (Libero, 1997, Mackay and Hein, 1989, Jutila, 1996, and Jutila 1998). In the context of trafficking, γδ T cells have been shown in many animals to exclude secondary lymphoid tissue during normal circulation throughout the body and instead preferentially recirculate through extralymphoid sites, such as the skin, gut mucosa, reproductive tract, and sites of inflammation (Jutila, 1998). Only recently have studies been done to compare the molecular mechanisms used by γδ T cells and other lymphocytes to migrate throughout the body and begin to address the basis for their unique trafficking behavior.
The process of trafficking involves recognition of the vascular lining of vessels in tissues by the circulating leukocyte, resulting in a sequential multistep process characterized by 1) rolling of the leukocyte along the vessel wall, 2) tight adhesion and arrest, and 3) transendothelial cell migration (Springer, 1994). Once leukocytes tightly adhere to the vessel wall, they can serve as adhesive substrates for continued recruitment of new leukocytes (Bargatze et al., 1994, Walcheck et al., 1996, Tu et al., 1996, and Alon et al., 1996). Most leukocyte-on-endothelial cell and leukocyte-on-leukocyte rolling interactions are mediated by a family of adhesion proteins called selectins (Springer, 1994, Tedder et al., 1995, and Jutila and Kurk, 1996). Three selectins have been defined: L-selectin on most leukocytes, E-selectin on inflamed endothelium, and P-selectin on activated platelets and inflamed endothelium (Tedder et al., 1995, and Jutila and Kurk, 1996). L-selectin mediates leukocyte rolling on endothelial cell monolayers as well as leukocyte-on-leukocyte rolling (Tedder et al., 1995). E- and P-selectin also support rolling interactions, but only at the level of the endothelium or immobilized platelets (Tedder et al., 1995, and Jutila and Kurk, 1996).

A number of years ago, we began a comparison of the trafficking mechanisms used by γδ T cells and other lymphocytes with an emphasis on selectin-mediated rolling (Jutila and Kurk, 1996, Jutila et al., 1994, and Jutila et al., 1997). We have used the bovine model for these studies because newborn calves, which provide homogenous lymphocyte populations with respect to their state of activation and memory cell status, can be
utilized. Also, for reasons undefined, γδ T cells are the predominant population of circulating T cells in newborn calves. Therefore, large numbers of cells are easily isolated for study. We have found that bovine γδ T cells exhibit a much greater capacity to roll on monolayers of endothelial cells, platelets, or recombinant E-selectin than other lymphocytes in assays done under physiologic flow (Jutila and Kurk, 1996, Jutila et al., 1994, and Jutila et al., 1997). Furthermore, we have shown that γδ T cells avidly roll on other immobilized γδ T cells, and rolling is completely blocked by anti-L-selectin mAbs (Jutila and Kurk, 1996). Finally, we found that bovine γδ T cells express 2-5 times the level of L-selectin as other lymphocytes; whereas, the expression of other adhesion molecules, such as the CD18 integrins, does not differ (Walcheck and Jutila, 1994). Here, we have used electron microscopy (EM) to further compare bovine γδ and αβ T cells to determine whether the increased capacity of γδ T cells to interact with endothelial cells and other T cells correlates with possible differences in the physical properties of these cells.

Materials and Methods

Animals

One-week to three-month-old Holstein calves, housed in the Montana State University large animal facility, were used as sources of peripheral blood.
Monoclonal Antibodies

The anti-L-selectin mAbs DREG 56, DREG 200, EL-246, and GD 4.22 have been described elsewhere (Kishimoto et al., 1990, Jutila et al., 1992, and Jutila and Kurk, 1996). Anti-WCl mAb IL-A29 has also been described elsewhere (American Tissue Type Culture Collection, Manassas, VA). FITC-conjugated goat anti-mouse antibodies were used to visualize primary antibody staining of L-selectin in the flow cytometry studies (Jackson ImmunoResearch, West Grove, PA).

Cell Lines

Human E-selectin transfectants were made by transfecting mouse L-cells with human ELAM (E-selectin) cDNA as previously described (Kishimoto et al., 1991), and were grown to confluency in T-175 flasks for γδ T cell isolation, as described (Walcheck et al., 1993).

Isolation of γδ and αβ T Cells

Gamma delta T cells were isolated, as described (Walcheck et al., 1993). Briefly, peripheral blood was collected from Holstein calves into heparin vacutainer tubes by jugular venipuncture. The blood was diluted 1:1 with Hank’s Balanced Salt Solution (HBSS, Sigma, St. Louis, MO), underlayed with 15 mL of Histopaque™, and centrifuged for 30 min at 700g. The buffer coat layer of PBMC’s was collected and incubated on plastic in Dulbecco’s Modified Eagle Medium (DMEM, Sigma) containing 0.7% BSA.
(Sigma) for 30 min at 37°C. Non-adherent lymphocytes were collected and incubated on confluent layers of E-selectin transfectants for 30 min as described (Walcheck et al., 1993). Gamma delta T cells specifically bind to E-selectin in a calcium-dependent manner whereas αβ T cells do not (Walcheck et al., 1993). Non-E-selectin adherent cells were poured off, the monolayers washed, and the γδ T cells isolated by incubation with 2 mM EDTA. This procedure yielded >95% γδ T cells, as analyzed by their expression of γδ TCR (Walcheck et al., 1993). The non-E-selectin adherent cells contained >85% αβ T cells as determined by flow cytometry (data not shown). Both lymphocyte subsets were collected and prepared for electron microscopy.

**Scanning Electron Microscopy**

Bovine lymphocytes were prepared as described above and scanning electron microscopy performed as described (Speer et al., 1979). Briefly, lymphocytes were adhered to 12 mm glass cover slips (Ted Pella, Redding CA) coated with CellTak® (100 µg/mL, Collaborative Biomedical Products, Bedford, MA). Adherent cells were fixed in 2.5% (v/v) glutaraldehyde in Millonig's phosphate buffer overnight, dehydrated, critical point dried, sputter coated with gold-palladium, and examined with a JEOL 100CX scanning/transmission electron microscope (Speer et al., 1979).
Transmission Electron Microscopy

Transmission electron microscopy (TEM) studies were done as described (Speer et al., 1997). Briefly, bovine lymphocytes were isolated and fixed in modified Karnovsky’s fixative overnight. Samples were partially dehydrated in ethanol, prestained with 1% (w/v) phosphotungstic acid (Ted Pella) and 1% (w/v) uranyl acetate (Ted Pella) in 70% ethanol, completely dehydrated and embedded in Spurrs' (Ted Pella) medium. Ultrathin sections were stained with lead citrate and examined by TEM.

Flow Cytometry

Flow cytometry was performed, as previously described (Walcheck et al., 1993a). Briefly, lymphocytes were collected, suspended at 1x10⁷ cells/mL, and 100 μL aliquots transferred into FACS tubes (Fisher Scientific Co., Pittsburgh, PA) for analysis. The mAbs described above were added to the samples at a concentration of 50 μg/mL and incubated on ice for 30 min, washed in PBS containing horse serum and sodium azide (FACS buffer). FITC-conjugated goat anti-mouse second stage was added and the cells incubated for an additional 30 min on ice. The cells were washed a final time, resuspended in FACS buffer, and analyzed on either a FACScan or FASCalibur (Becton Dickenson). 10-20,000 events were collected for each sample.
Results

Scanning Electron Microscopy of Bovine αβ and γδ T Cells

By scanning electron microscopy (SEM), we found that γδ T cells have more microvilli (i.e., folds or projections from the plasmalemma) on a per cell basis than αβ T cells (Figure 2.1 versus Figure 2.2). Gamma delta T cells had 59±1.3 (n=19) microvilli/cell whereas αβ T cells had 28±1.8 (n=18) microvilli/cell. A blind count was employed to confirm our observation. Surprisingly, γδ T cells were ~32 % smaller than αβ T cells (3.9±0.01 mm versus 5.7±0.01 mm; n=33 and n=26 respectively; Figure 2.1, Panel B versus Figure 2.2, Panel B). All data are representative of 3 different preparations from 3 different Holstein calves.

Transmission Electron Microscopy of Bovine αβ and γδ T Cells

We also examined the expression of L-selectin on bovine lymphocytes to determine if, as shown with human leukocytes (Picker et al., 1991, and Bruehl et al., 1996), L-selectin is localized to the tips of microvilli. As found for human neutrophils and lymphocytes, L-selectin was localized at the tips of microvilli of bovine lymphocytes (Figure 2.3, Panel A). Little or no L-selectin was detected on the rest of the plasmalemma of γδ T cells, concurrent with the data previously reported for human leukocytes (Picker et al., 1991, and Bruehl et al., 1996). In contrast, WC1, a γδ T cell subset marker,
Figure 2.1. (A) Low-magnification SEM showing the uniform size of several \( \gamma \delta \) T cells; bar = 5 \( \mu \)m; original magnification X6,000. (B) Higher magnification SEM of a \( \gamma \delta \) T cell showing numerous microvilli (Mv); bar = 1 \( \mu \)m; orig. mag. X20,000.

Figure 2.2. (A) Low-magnification SEM showing several \( \alpha \beta \) T cells with a few platelets (Pt); bar = 5 \( \mu \)m; orig. mag. X6,000. (B) Higher magnification SEM of an \( \alpha \beta \) T cell showing microvilli (Mv); bar = 1 \( \mu \)m; orig. mag. X20,000.
Figure 2.3. (A) TEM of a γδ T cell treated with DREG 56 (anti-L-selectin) plus colloidal gold-conjugated antibody; note gold label at tips of microvilli (arrows); bar = 1 μm; orig. mag. X20,000. (B) TEM of WC1 (anti-γδ T cell) labeled with colloidal gold on the plasmalemma (Pl) of a γδ T cell; Mv, microvilli; bar = 0.5 μm; orig. mag. X70,000.

Figure 2.4. (A) TEM showing localization of L-selectin on two adjacent bovine γδ T cells by DREG 56 and colloidal gold-conjugated antibody; note that L-selectin is localized primarily at the tips of the microvilli of adjacent cells; bar = 0.5 μm; Nu, nuclei; *, extracellular space; orig. mag. X70,000. (B) High-magnification TEM of the tips of microvilli of two γδ T cells in close proximity showing colloidal gold-labeled L-selectin; bar = 0.1 μm; orig. mag. X150,000.
localized on the plasmalemma covering the γδ T cell body but was absent on microvilli (Figure 2.3, Panel B). A representative micrograph of two γδ T cells in close proximity is also shown in Figure 2.4, Panel A. In the same figure, Panel B shows a high magnification (X150,000) of two microvilli in contact and a cluster of L-selectin localized at the tips of the microvilli.

Flow Cytometric Analysis of L-selectin on αβ Versus γδ T Cells

Finally, we have previously shown by flow cytometry that γδ T cells have more L-selectin on their surface than αβ T cells by DREG 56 mAb staining (Walcheck and Jutila, 1993a). This finding was reconfirmed here. Four mAbs, which stain distinct epitopes on L-selectin, all stained brighter on γδ versus αβ T cells (Figure 2.5). Thus, there clearly is more L-selectin on bovine γδ T cells, which we now hypothesize is due to an increased number of microvilli on these cells.

![Figure 2.5. Mode fluorescence anti-L-selectin staining of αβ versus γδ T cells by four distinct anti-L-selectin mAbs. All four mAbs stained γδ T cells brighter than αβ T cells.](image-url)
Discussion

Our in vitro studies have shown that \(\gamma\delta\) T cells are far more efficient at initiating adhesive rolling interactions on both endothelial and adherent leukocyte monolayers under shear forces comparable to those found in blood flow in vivo (Jutila and Kurk, 1996, Jutila et al., 1994, and Jutila et al., 1997). This may be due, in part, to differences in expression levels of adhesion molecules on \(\gamma\delta\) T cells, but the observations shown here provide the basis for an alternative explanation. Increased numbers of microvilli, where some adhesion molecules are located, would effectively increase the number of contact sites, thus enhancing the opportunity for adhesion to occur. The smaller size of the \(\gamma\delta\) T cell may also be a benefit. Due to its lower profile, the \(\gamma\delta\) T cell would not experience the same shear forces in blood flow as \(\alpha\beta\) T cells. Thus, three differences have been defined which likely contribute to the increased efficiency of bovine \(\gamma\delta\) T cell/endothelial cell interactions: these cells have been shown to 1) have increased expression of some adhesion molecules, 2) are smaller in size, and 3) have considerably more surface microvilli to initiate cell/cell contact.

In summary, these results further define the differences between bovine \(\gamma\delta\) and \(\alpha\beta\) T cells. Previously, these cell types could be distinguished by their T cell receptors for antigen (TCRs), expression of CD4/CD8 on the \(\alpha\beta\) T cell and other lineage-specific antigens, such as WC1, on the \(\gamma\delta\) T cell. Differences in the size and surface topography
of γδ and αβ T cells may be important in the interaction of these cells with other host cells, such as endothelial cells, or targets they intend to kill, such as tumor cells or infectious agents.
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CHAPTER 3

ANTIBODY BINDING TO A CONFORMATION-DEPENDENT EPITOPE INDUCES L-SELECTIN LINKAGE TO THE CYTOSKELETON

Introduction

Recruitment of leukocytes from the blood into tissues is controlled by a variety of adhesion molecules on the surface of the endothelium and circulating leukocytes, one of which is L-selectin (Butcher et al., 1999). L-selectin is important in the normal migration and circulation of both memory and naive lymphocytes (Gallatin et al., 1983 and Steeber et al., 1996a, and 1996b) and is central to leukocyte-endothelial cell interactions, including neutrophil rolling along the vessel wall (von Andrian et al., 1991). We, and others, have also shown that L-selectin and corresponding ligands are important in leukocyte-on-leukocyte rolling interactions that may amplify further recruitment of leukocytes from the blood into tissues (Bargatze et al., 1994a, Jutila and Kurk, 1996, Fuhlbrigge et al., 1996, Alon et al., 1996 and Walcheck et al., 1996). A common feature of all adhesive interactions mediated by L-selectin is that they occur under flow in the bloodstream. Indeed, threshold levels of shear promote adhesion through L-selectin (Finger et al., 1996, and Lawrence, et al, 1997).

L-selectin is constitutively expressed on most circulating leukocytes, but is uniquely regulated when the cell becomes activated. Following activation of lymphocytes or myeloid cells with chemotactic factors or phorbol esters, there is a rapid increase in
functional avidity (Spertini et al., 1991) which, under most circumstances, is followed by proteolytic cleavage of the protein from the cell surface (Kishimoto, et al., 1989, Griffin, et al., 1990 and Smith, et al., 1991). The basis for the increased functional activity in L-selectin immediately following activation of the leukocyte is poorly understood. It may be due to phosphorylation of L-selectin or other proteins, dimerization, hyperthermic conditions, cytoskeletal association of L-selectin through its cytosplasmic tail, or conformational changes in the protein (Haribabu, et al., 1997, Li et al., 1998, Wang et al., 1998 and Evans et al., 1999). Endoproteolytic release of L-selectin from the surface of leukocytes is regulated by structural features of the L-selectin protein (Chen, et al., 1995, Migaki et al., 1995). Calmodulin, an intracellular calcium regulatory protein, specifically co-precipitates with L-selectin through a direct association with the cytoplasmic tail and calmodulin inhibitors disrupt L-selectin-dependent adhesion by inducing proteolytic release of L-selectin from the cell surface (Kahn et al., 1998).

Many reports suggest that L-selectin can also function as a signal transduction molecule. Crosslinking of human L-selectin with mAbs leads to neutrophil activation as measured by Ca\textsuperscript{2+} flux, superoxide generation, increased adhesiveness, and activation of intracellular protein pathways, such as tyrosine phosphorylation and MAP kinase production (Po et al., 1995, Waddell et al., 1995, Crockett-Torabi et al., 1995, Simon et al., 1995, Sikorski et al., 1996, and Gopalan et al., 1997). Crosslinking of L-selectin also potentiates the response of neutrophils to formyl peptides (Waddell et al., 1994). In most studies, crosslinking of L-selectin using primary anti-L-selectin mAb followed by a
secondary reagent is requisite for signaling, though important exceptions exist. For example, signaling through L-selectin can also be induced by sulfatides, which bind directly to L-selectin (Laudana et al., 1994). Further, a mAb directed against a highly conserved region of L-selectin can signal and cause increased adhesion of lymphoid cells transfected with human L-selectin, neutrophils, and lymphocytes, in the absence of a crosslinking secondary antibody (Steeber et al., 1997). Therefore, it is clear that L-selectin can act as a signaling molecule under certain conditions.

The cytoplasmic tail of L-selectin is vital to its function during leukocyte rolling and adhesion (Kansas et al., 1993). Deletion mutants lacking the carboxyl terminal 11 amino acids of the cytoplasmic tail of L-selectin do not bind to HEV and do not establish rolling interactions in vivo (Kansas et al., 1993). Subsequent work demonstrated a direct link between the cytoplasmic tail of L-selectin and the cytoskeletal proteins α-actinin and vinculin (Pavalko et al., 1995) and recently, L-selectin was shown to dynamically associate with the cytoskeleton (Evans et al., 1999). Antibody crosslinking, hyperthermic treatment, and ligand binding studies demonstrate that the cytoplasmic tail is important to the function of L-selectin by regulating linkage to the actin cytoskeleton through direct binding of the cytoplasmic tail (Evans et al., 1999). However, the nature of the linkage has not been clearly shown and it is not known what predisposes L-selectin to link to the cytoskeleton.
Here, we have examined the effect of treating leukocytes with an anti-L-selectin mAb that recognizes a highly conserved and functionally important epitope on L-selectin expression. We show that one mAb (LAM1-116), which binds an epitope in the lectin domain (Steeber et al., 1997), causes a structural change in human, bovine, and ovine L-selectin in the absence of cellular activation that is detected by increased staining of a second anti-L-selectin mAb, EL-246. The induced conformation predisposes L-selectin to link to the cytoskeleton. As both mAbs bind functional epitopes on L-selectin, this type of structural regulation may be important in L-selectin function.

Materials and Methods

Animals

One-week to three-month-old Holstein calves, and eighteen-month-old sheep, housed in the Montana State University large animal facility, and healthy human donors were used as sources of peripheral blood.

Cell Preparations

Peripheral blood was collected by venipuncture into citrate or heparin anticoagulant tubes as previously described (Jutila et al., 1992). Total leukocytes were harvested using a hypotonic solution for 10 sec followed by rapid dilution in either HBSS or PBS (Sigma, St. Louis, MO) and centrifuged at 200g for 5 min. The process was repeated as necessary to rid preparations of red blood cells.
Monoclonal Antibodies

The following mAbs were used. Anti-L-selectin mAbs included DREG 55, DREG 56, DREG 110, DREG 152, and DREG 200 (Kishimoto et al., 1990); LAM1-1, LAM1-5, LAM1-101, LAM1-102, LAM1-104, LAM1-108, LAM1-110, LAM1-115, LAM1-116, LAM1-118, LAM1-119, LAM1-120, LAM1-126 (Steeber et al., 1997), EL-246 and GD 4.22 (Jutila, 1992 and 1996), and Leu-8 (Becton Dickenson, Mountain View, CA). Anti-CD18 mAbs included R15.7 (gift from R. Rothlein), IB4 (ATCC, Manassas, VA: ATCC #HB-10164), and MHM-23 (Dako, Carpinteria, CA). Anti-CD11b mAb Leu-15 (Becton Dickenson) was also used. Other mAbs included HECA 452 [anti-CLA (Picker et al., 1990)], Hermes-3 [anti-CD44 (Picker et al., 1989)], GD 3.5 [anti-γδ T cell (unknown γδ T cell specific marker, Jones et al., 1996)], GD 3.8 [anti-γδ TCR (Wilson et al, 1998)], and EL-112 [anti-E-selectin (Bagatze et al., 1994b)].

Cell Lines

The mouse pre-B 300.19 cells transfected with either full length human L-selectin cDNA (300.19/L-selectin) or a deletion mutant lacking the carboxyl-terminal 11 amino acid residues of the cytoplasmic domain (300.19/LΔcyto) have been previously described (Kansas et al., 1993). The selectin transfectants used in the antibody mapping studies were described elsewhere (Tu et al., 1996).
Flow Cytometry of Non-detergent-treated Cells

Isolation of leukocytes and flow cytometric analysis were as described (Jutila et al., 1992, and Bargatze et al., 1994b). Briefly, 1x10^6 leukocytes were incubated with 1 µg of LAM1-116 or other mAbs at 37°C or 4°C, 10-100 mM sodium azide (Sigma Co.), 1-100 mM Herbimycin A (Calbiochem), Genistein (Calbiochem), or calpeptin (Calbiochem) for 15 min, or buffer alone. After incubation with mAb, the cells were placed on ice and FITC-conjugated (Molecular Probes, Eugene, OR) EL-246, biotin-conjugated (Pierce, Rockford IL) EL-246, or other FITC- or PE-conjugated mAbs were added. Cells were incubated with mAbs for 30 min on ice, washed in PBS with horse serum (FACS buffer), and staining measured on either a FACScan or FACSCalibur (Becton Dickenson, Mountain View, CA). Data were collected from 10,000 cells and mode fluorescence staining values reported in table form or as representative histograms. L-selectin levels were also measured by an indirect stain on the 300.19 L-selectin transfectants, as described (Jutila et al., 1992). LAM1-116 and DREG 56 Fab treatment of leukocytes was performed as above.

Flow Cytometry of Detergent-solubilized Cells

Flow cytometric analysis of L-selectin association with the detergent-insoluble cytoskeleton was as described (Geppert and Lipsky, 1991, and Nebe et al., 1997), with minor exceptions. Specifically, leukocytes were harvested and isolated as described above and incubated with LAM1-116 or other anti-L-selectin mAbs at 37°C for 15 min. The
cells were washed in FACS buffer and either treated with another anti-L-selectin mAb for 15 min at 37°C, or treated directly with 0.5% NP-40 lysis buffer (150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 2% Goat Serum, 0.5% NP-40) for 15 min at room temperature in the absence of antibody crosslinking, as described (Nebe et al., 1997). Mock buffer without NP-40 was used as a control. The cells were washed in FACS buffer and incubated with PE-labeled goat anti-mouse F(ab')₂ (Jackson ImmunoResearch Labs, Inc., West Grove PA) for 30 min on ice. Staining was measured by gating on the detergent-insoluble cytoskeletal fraction using forward and side scatter. The gated population was confirmed by phalloidin-FITC (Sigma), which specifically stains the detergent-insoluble cytoskeleton (Nebe et al., 1997).

**Generation of LAM1-116 and DREG 56 Fab**

Monovalent Fab fragments were generated from whole IgG molecules with papain by the ImmunoPure® Fab Preparation Kit from Pierce, per manufacturer’s instructions. To confirm Fab production, 8% SDS-PAGE gels were run under non-reducing and reducing conditions and molecular weight determined by Coomassie stain. In some cases, the Fab preparation was also incubated with Protein G beads (Boehringer Mannheim, Germany) to reduce the levels of contaminating Fc fragments.
Phase Contrast Fluorescence Microscopy

Bovine lymphocytes were isolated as described (Jones et al., 1996), treated with mAbs, incubated with 0.5% NP-40 lysis buffer for 10 min at room temperature, and stained with PE-labeled goat anti-mouse F(ab’)2 (Jackson ImmunoResearch) secondary antibody on ice for 30 min. Cells were washed in FACS buffer and incubated with phalloidin-FITC (Sigma) for 20 min on ice. Cells were washed a final time in FACS buffer and placed in 16-well, LabTek glass chamber slides (Nunc, Naperville, IL) for microscopic examination. Fluorescent microscopy was performed using a super high pressure mercury lamp power supply (Nikon, Melville, NY) model HB-1013AF, linked to a Nikon inverted microscope (Eclipse TE300), and digital data captured using a Spot digital imaging system (Diagnostic Instruments, Inc., Sterling Heights, MI). Results are visualized at 400X magnification. The fluorescence micrographs are overlays of red and green images to show double positive regions of cells (orange).

Immunoprecipitation of L-selectin and Densiometric Analysis of Band Intensities

MAb EL-246 was covalently linked to activated cyanogen-bromide (CNBr) Sepharose 4B beads (Pharmacia LKB, Uppsala, Sweden), which were then blocked with 1M glycine at 25°C for 3 hrs (Walcheck et al., 1993). Bovine lymphocytes were surface labeled with biotin (Pierce), as described (Jones et al., 1996), and detergent lysates prepared from these cells. The preparations were incubated with CNBr beads for 2 hrs at
25°C as a pre-clearing step (Jones et al., 1996). The lysates were drained from the CNBr columns and incubated without mAb, LAM1-116, other anti-L-selectin mAbs, or irrelevant mAbs at 37°C for 15 min. After incubation, an equal quantity of EL-246-labeled CNBr beads were added to each lysate and L-selectin was immunoprecipitated either at 4°C overnight or at 37°C for 2.5 hrs. The beads were washed 3x with wash buffer (Walcheck et al., 1993), mixed with reducing or non-reducing buffer, boiled for 3 min, and loaded directly onto an 8% polyacrylamide gel. Gels were electrophoresed and the proteins were transferred to PVDF membrane (Bio-Rad, Hercules, CA) overnight at 4°C. Proteins were visualized using a streptavidin horseradish peroxidase (Amersham, Buckinghamshire, England) reaction and ECL detection system (Amersham) and developed on X-OMAT (Kodak) film. Densiometric analysis was performed on the intensity of the immunoprecipitated L-selectin bands using 1-D MULTI on an Alpha Innotech Corp. IS-1000 Digital Imaging System. Protein G (Boehringer Mannheim, Germany) immunoprecipitation using anti-L-selectin mAb GD 4.22 was used as a positive control for L-selectin immunoprecipitation, as described (Jones et al., 1996).

Results

MAb LAM1-116 Enhances Leukocyte Staining of L-selectin by mAb EL-246

Pretreatment of human neutrophils or bovine lymphocytes with mAb LAM1-116 at 37°C for 15 minutes enhanced their subsequent staining of L-selectin by FITC-labeled
Figure 3.1. LAM1-116 treatment of human or bovine leukocytes at 37°C increases the presentation of a functionally conserved epitope on L-selectin as measured by another directly FITC-labeled mAb, EL-246. Representative overlay histograms are shown in Panel A: EL-246 alone (dotted line), EL-246 following pretreatment with LAM1-116 (thick solid line). Panel B represents staining of human neutrophils and bovine lymphocytes with and without LAM1-116 treatment. Each panel is representative of over 10 separate experiments.

*A paired students t test was performed on the untreated vs. LAM1-116 treated cells for both human neutrophils and bovine lymphocytes, p<0.005.
Figure 3.2. LAM1-116 specifically increases the presentation of the EL-246 epitope. Other isotype-matched and mismatched anti-L-selectin mAbs directed against distinct epitopes are not enhanced by LAM1-116 pretreatment (Panel A). MAbs directed against other cell surface antigens do not enhance the presentation of the EL-246 epitope (Panel B). Data is representative of 3 separate experiments and the S.E.M. shown.
EL-246 mAb, as measured by flow cytometric analysis (Figure 3.1A, and B). The same effect was seen with all leukocyte cell types tested from humans, cattle, and sheep (data not shown). The increased staining by EL-246 after leukocyte treatment with the LAM1-116 mAb was dose-dependent with 1 µg/ml of LAM1-116 mAb giving optimal results (data not shown). Importantly, the increase in EL-246 staining was not due to increased expression of L-selectin protein, since the staining of four other FITC- or PE-labeled anti-L-selectin mAbs was unaffected (Figure 3.2A). This effect was also detected by EL-246 labeled with biotin (Table 3.1). Moving human or bovine leukocytes from 4°C to 37°C for 15 minutes greatly reduced the staining of L-selectin by EL-246 in the absence of L-selectin shedding (Table 3.1, and data not shown). In contrast, staining by other anti-L-selectin mAbs was not altered by this treatment (data not shown).

To determine the specificity of the LAM1-116 mAb-induced upregulation of the EL-246 epitope, human and bovine leukocytes were pretreated with 19 other anti-L-selectin mAbs, including at least 6 reactive with the same domain as LAM1-116, as well as mAbs directed to other surface antigens, including R15.7 (anti-CD18), HECA 452 (anti-CLA), Hermes-3 (anti-CD44), GD 3.5 (unknown ligand on γδ T cells) and GD 3.8 (anti-γδ TCR). None of the other anti-L-selectin mAbs or other mAbs listed above induced increased EL-246 staining [Figures 3.2A (example of 4 other anti-L-selectin mAbs) and 3.2B and data not shown]. In similar experiments, LAM1-116 pretreatment had no effect on the expression of other surface antigens, such as CD18 and TCR (data
not shown). To be confident staining of L-selectin by FITC-labeled EL-246 was specific, excess (10x) unlabeled EL-246 or excess isotype-matched (10x) GD 3.8 (anti-γδ TCR) antibodies were added along with FITC-labeled EL-246, as above. The addition of excess unlabeled EL-246 mAb effectively blocked the staining of FITC-labeled EL-246 (Table 3.1). However, treatment with excess GD 3.8 mAb did not inhibit staining of FITC-labeled EL-246 to an appreciable extent (Table 3.1).

Table 3.1. Expression of the EL-246 Epitope on Leukocytes Under Various Conditions

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>EL-246 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>100%</td>
</tr>
<tr>
<td>37°C</td>
<td>50% ± 7%</td>
</tr>
<tr>
<td>LAM1-116</td>
<td>270% ± 20%*</td>
</tr>
<tr>
<td>LAM1-116**</td>
<td>250% ± 6%**</td>
</tr>
<tr>
<td>LAM1-116 with excess unlabeled EL-246</td>
<td>4% ± 4%</td>
</tr>
<tr>
<td>LAM1-116 with excess unlabeled GD 3.8</td>
<td>240% ± 9%</td>
</tr>
<tr>
<td>LAM1-116 with 50 mM sodium azide</td>
<td>220% ± 5%</td>
</tr>
<tr>
<td>LAM1-116 with 50 μM Herbinycin A</td>
<td>225% ± 7%</td>
</tr>
<tr>
<td>LAM1-116 with 50 μM Genistein</td>
<td>235% ± 9%</td>
</tr>
</tbody>
</table>

Level of EL-246 expression, measured by EL-246 staining, was normalized to 100% for each experiment and the mean ± S.E.M. listed.
All treatments were done at 37°C unless otherwise stated. At least 3 separate experiments were done for all the treatments listed.
*The amount of EL-246 epitope enhancement varied depending upon the amount of γδ T cells present in the preparations as γδ T cells express 2-5 fold more L-selectin than αβ T cells.
**The prior readout was EL-246-FITC and these numbers are for EL-246-Biotin.
LAM1-116 Fab also Induced an Increase in Expression of the EL-246 Epitope

To determine whether intact LAM1-116 was requisite for enhanced EL-246 reactivity, a Fab fragment of LAM1-116 was generated by papain digestion, as described in Materials and Methods. The Fab fragment of LAM1-116 was able to enhance EL-246 epitope expression, although to a lesser extent than the whole IgG antibody (Figure 3.3). This data suggested that LAM1-116 did not induce crosslinking of adjacent L-selectin molecules as monovalent Fab fragments do not have a second binding site needed for effective crosslinking. The larger increase in EL-246 staining seen with the IgG was likely due to the ability of dual binding sites to act upon L-selectin and enhance the EL-246 epitope more effectively. To ensure the specificity of the LAM1-116 Fab interaction, Fab fragments of DREG 56 mAb were generated, and although the antibody preparations stained L-selectin, they did not increase EL-246 reactivity in parallel assays (data not shown).

![EL-246 Staining](image)

Figure 3.3. LAM1-116 monovalent Fab enhances the presentation of the EL-246 epitope on bovine lymphocytes. Leukocytes from human and bovine preparations were enhanced by LAM1-116 Fab treatment (data not shown). Data are representative of 3 separate experiments and the S.E.M. shown.
The Tyrosine Kinase Inhibitors Herbimycin A and Genistein do not Block LAM1-116 Upregulation of the EL-246 Epitope

Tyrosine kinase activity may play a role in the function of L-selectin (Waddell et al., 1995). To address whether tyrosine kinases are involved in upregulation of the EL-246 epitope, we tested the effects of two tyrosine kinase inhibitors, Herbimycin A and Genistein, to see if they could block the increased presentation of the EL-246 epitope in the presence of the LAM1-116 mAb. As shown in Table 3.1, neither Herbimycin A or Genistein blocked the LAM1-116 enhancement of the EL-246 epitope. No inhibition of EL-246 epitope enhancement was seen over a range of 1-100 mM of each inhibitor. Thus, tyrosine kinase activity seems to have no role in the conformational change induced in L-selectin by the LAM1-116 mAb. To further investigate whether other signaling events in the cell were responsible for the upregulation of the EL-246 epitope, studies were undertaken at 4°C, and in the presence of sodium azide, which blocks electron transport and eventual ATP production. Both treatments had no inhibitory effect on the LAM1-116 mAb’s ability to induce the conformation that increases the expression of the EL-246 epitope (Table 3.1). Therefore, enhancement of the EL-246 epitope is not dependent upon common signaling events.
Expression of the EL-246 Epitope on Recombinant Selectins Generated by Swapping Different Domains of L-, E-, and P-selectin

EL-246 mAb recognizes a conserved epitope on both E- and L-selectin and original mapping studies using recombinant chimeric selectins comprised of L- and P-selectin extracellular domains demonstrated that the SCR domain of L-selectin was important for optimal EL-246 binding (Jutila et al., 1992). Additional chimeric selectin molecules were generated, expressed and analyzed for EL-246 staining to gain further insight into the nature of the EL-246 epitope. As expected, EL-246 recognized recombinant L and E-selectin (Table 3.2, LLL and EEE), but not P-selectin (data not shown). Staining of multiple chimeric molecules, generated by swapping different domains of each selectin, failed to provide a precise location of the EL-246 epitope. The EL-246 epitope did not reside in the SCR domain of L-selectin, since EL-246 failed to stain a construct containing the lectin domain of P-selectin, and EGF and SCR domains of L-selectin (Table 3.2, PLL). Weak reactivity was seen with a construct containing the lectin domain of L-selectin, and EGF and SCR domains of P-selectin (LPP). Replacing the SCR domain in this latter construct with an SCR from L-selectin increased staining slightly, but not nearly as much as seen in our earlier study (Jutila et al., 1992), suggesting variability in expression of the EL-246 epitope on the same transfectants analyzed at different times. The lectin domain of L-selectin did not confer EL-246 reactivity, even if combined with EGF or SCR domains of E-selectin (Table 3.2, LEE and LLE). The greatest amount of EL-246 staining was seen with a chimera expressing the lectin and
EGF domains of E-selectin and SCR domain of L-selectin (Table 3.2, EEL). From these stains we conclude that the EL-246 epitope is not encoded by a specific domain of L-selectin, but whose expression is dependent upon a proper conformation of the molecule that can be altered by changes in one or more of its domains.

Table 3.2. EL-246 Staining of Chimeric Recombinant Selectin Transfectants.

<table>
<thead>
<tr>
<th>Chimeric Selectin</th>
<th>EL-246</th>
<th>LAM1-14 (SCR)</th>
<th>LAM1-3 (Lectin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.2</td>
<td>3.0</td>
<td>3.4</td>
</tr>
<tr>
<td>LLL</td>
<td>18.4</td>
<td>67.4</td>
<td>161</td>
</tr>
<tr>
<td>LEE</td>
<td>3.2</td>
<td>3.0</td>
<td>20</td>
</tr>
<tr>
<td>ELL</td>
<td>60.8</td>
<td>26.4</td>
<td>2.9</td>
</tr>
<tr>
<td>EEE</td>
<td>21.3</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>EEL</td>
<td>142</td>
<td>57.3</td>
<td>2.8</td>
</tr>
<tr>
<td>LLE</td>
<td>3.9</td>
<td>16.4</td>
<td>34</td>
</tr>
<tr>
<td>LLP</td>
<td>2.8</td>
<td>2.6</td>
<td>11.3</td>
</tr>
<tr>
<td>LPP</td>
<td>6.2</td>
<td>2.9</td>
<td>74</td>
</tr>
<tr>
<td>PLL</td>
<td>2.8</td>
<td>19.7</td>
<td>2.5</td>
</tr>
<tr>
<td>LPL</td>
<td>7.2</td>
<td>33</td>
<td>60</td>
</tr>
</tbody>
</table>

EL-246 staining of L-, E-, and P-selectin chimeras was analyzed by flow cytometry. LAM1-14 and LAM1-3 mAb staining is also shown as a comparison to EL-246. Mean fluorescence intensity is reported. Staining of the parent cell lines is reported in the row titled None. Data was generated by Dr. Doug Steeber at Duke University.

Treatment of Detergent Lysate Preparations with LAM1-116 mAb Specifically Enhances L-selectin Immunoprecipitation by EL-246 mAb Covalently Linked to CNBr Beads

To test whether or not the intact leukocyte was needed for a conformational change to take place in the L-selectin protein, bovine lymphocytes were surface labeled with biotin and detergent lysates prepared. The biotin-labeled lysates were divided into
fractions, which were treated with the LAM1-116 mAb, a negative control antibody, or no antibody at all, for 15 minutes at 37°C. The treated lysates were then immunoprecipitated with EL-246 covalently attached to Sepharose 4B beads either at 4°C or 37°C. As shown in Figure 3.4A, lane 2, EL-246 weakly immunoprecipitated L-selectin from control lysates. By contrast, treatment of lysates with LAM1-116 increased the amount of L-selectin subsequently immunoprecipitated by EL-246 when compared to the negative control, an irrelevant, isotype-matched mAb, EL-112 (Figure 3.4A, lanes 3 and 4). Other anti-L-selectin mAbs, including DREG 56, were tested, and only LAM1-116 caused increased immunoprecipitation of L-selectin (data not shown). Protein G bead immunoprecipitation of L-selectin with anti-L-selectin mAb GD 4.22 was used as a control for L-selectin precipitation (Figure 3.4A, lane 1). Densiometric analysis was employed to quantify the differences in band intensity. Analysis of the bands showed that LAM1-116 mAb treatment of the detergent lysates increased the amount of L-selectin immunoprecipitated by EL-246 mAb four-fivefold compared to non-treated or other mAb treated fractions (Figure 3.4B, lanes 2-4).

EL-246 mAb Can Induce L-selectin Cytoskeletal Association in the Presence of LAM1-116

Recently, L-selectin was shown to dynamically associate with the actin cytoskeleton under a variety of conditions (Evans et al., 1999). To investigate L-selectin cytoskeletal interactions, cells were treated with primary anti-L-selectin mAbs as
Figure 3.4. LAM1-116 treatment increases the amount of L-selectin immunoprecipitated from bovine lysates by EL-246 covalently linked to activated CNBr beads. Panel A represents a SDS-PAGE gel with L-selectin immunoprecipitation. Lane 1, protein-G control immunoprecipitation of L-selectin with anti-L-selectin mAb, GD 4.22. Lane 2, untreated lysate immunoprecipitated with EL-246-labeled CNBr beads. Lane 3, LAM1-116 treated lysate immunoprecipitated with EL-246 beads. Lane 4, EL-112 treated lysate immunoprecipitated with EL-246 beads (irrelevant, isotype-matched anti-E-selectin mAb). Panel B, densiometric analysis of lanes 2-4 in Panel A. LAM1-116-treated lysate was normalized to 100% L-selectin immunoprecipitation. Data are representative of 3 separate experiments and the S.E.M. shown.

described in Materials and Methods, subjected to 0.5% NP-40 buffer, and L-selectin staining of the detergent-insoluble cytoskeleton visualized by goat F(ab')2 anti-mouse antibody labeled with phycoerythrin. Thus, if cytoskeletal association of L-selectin was detected, it was due to a direct effect of the mAbs on L-selectin and not crosslinking since the cells were treated with detergent prior to the addition of the secondary antibody. This is in contrast to the recent report by Evans and colleagues (1999) in which crosslinking by second stage antibody was a prerequisite to antibody-induced cytoskeletal association of L-selectin.
Treatment of human and bovine lymphocytes with the LAM1-116 or the EL-246 mAbs alone did not induce association of L-selectin with the cytoskeleton (Figure 3.5A). However, in the presence of LAM1-116, EL-246 induced L-selectin association with the cytoskeleton without subsequent crosslinking of the surface protein (Figure 3.5B). Other anti-L-selectin mAbs tested did not induce L-selectin association in tandem with LAM1-116 mAb treatment, suggesting that EL-246 mAb cytoskeletal induction was specific to the LAM1-116/EL-246 interaction with L-selectin (data not shown). Crosslinking studies parallel to those done by Evans et al. (1999) did show L-selectin cytoskeletal association but the association was no more dramatic than LAM1-116/EL-246 antibody treatment alone (data not shown).

Cytoskeletal association of L-selectin was also visualized by two-color fluorescent microscopy with PE-labeled secondary antibody and phalloidin-FITC, which specifically stains the actin cytoskeleton (Nebe et al., 1997). Parallel to our flow cytometry results, engagement of the EL-246 epitope in the presence of the LAM1-116 mAb caused L-selectin to associate with the cytoskeleton (Figure 3.6B). The micrographs are overlays of a red (L-selectin) and a green (phalloidin-FITC, actin cytoskeleton) digital image. A phase contrast micrograph of detergent-treated cells, matching the micrograph in 3.6B, is shown in 3.6A. Phalloidin-FITC staining was specific as it did not stain viable, non-detergent-treated cells (data not shown). Also, non-detergent-treated lymphocytes exhibited a normal, punctate L-selectin staining pattern (data not shown). As seen with our flow cytometry data (Figure 3.5), neither the LAM1-
Figure 3.5. In the presence of LAM1-116, EL-246 can induce association of L-selectin with the actin cytoskeleton. Panel A, LAM1-116 or EL-246 alone do not induce L-selectin cytoskeletal association [solid line, EL-246, dotted line, LAM1-116, stippled line, second stage goat anti-mouse F(ab')₂ PE]. Panel B, treatment of bovine lymphocytes with LAM1-116 predisposed L-selectin to cytoskeletal association, which in turn was triggered by EL-246 epitope engagement [solid line, EL-246 treatment in the presence of LAM1-116, dotted line, second stage goat anti-mouse F(ab')₂ PE alone]. Data are representative of 4 separate experiments.
Figure 3.6. Phase contrast fluorescent microscopy of bovine lymphocytes. Panel A, phase contrast micrograph of the fluorescent overlay in Panel B. Panel B, overlay of green (phalloidin-FITC) and red (anti-L-selectin mAbs LAM1-116 and EL-246 visualized with goat anti-mouse F(ab')\textsubscript{2} PE) stains showing cytoskeletal association of L-selectin (red and orange). Arrows represent examples of cytoskeletal association (red and orange) of L-selectin in the micrograph. Panel C, overlay of green (phalloidin-FITC) and red (LAM1-116). Notice the lack of red and/or orange staining demonstrating no cytoskeletal association of L-selectin with LAM1-116 alone. Panel D, overlay of green (phalloidin-FITC) and red (EL-246). Notice lack of red or orange staining with EL-246 alone. Data are representative of 4 separate experiments. Micrographs were taken at 400X magnification, except for Panels C and D; 200X, bar = 10 μm.
116 mAb nor the EL-246 mAb alone induced L-selectin association with the cytoskeleton (Figures 3.6C and D). These data suggest that the conformational change in L-selectin induced by the LAM1-116 mAb predisposes L-selectin to cytoskeletal association.

**Staining of L-selectin on Wild Type and LΔCyto Transfectants Lacking an Intact Cytoplasmic Tail**

Since the EL-246 epitope appears to require a unique conformation of L-selectin, we tested whether an intact cytoplasmic tail domain, which can link to the cytoskeleton, was required for optimal staining. L-selectin transfectants that lack the carboxyl terminal 11 amino acids of the cytoplasmic tail domain (LΔCyto) of L-selectin do not bind to HEV *in vitro* nor do they exhibit rolling interactions *in vivo* in exteriorized rat mesenteric venules, suggesting that the cytoplasmic domain of L-selectin regulates leukocyte adhesion by controlling cytoskeletal interactions and/or receptor avidity (Kansas et al., 1993). Staining of the EL-246 epitope on L-selectin in the LΔCyto versus the wild type transfectants was reduced (Figure 3.7A). Indeed, EL-246 staining of the majority of the LΔCyto mutant cells fell below the upper threshold of background staining. In comparison, LAM1-116 staining of the LΔCyto mutants remained high (Fig. 3.7B). To test whether or not LAM1-116 could enhance the expression of the EL-246 epitope on the cytoplasmic tail mutants, the mutants were pretreated with LAM1-116 parallel to the studies described above. Interestingly, EL-246 epitope expression could be enhanced by LAM1-116 pretreatment on both the wild type and the LΔCyto mutants (Figure 3.8A).
Figure 3.7. Staining of L-selectin with EL-246 and LAM1-116 on wild type and LΔCyto transfectants. Panel A, EL-246 staining of wild type and LΔCyto transfectants. Panel B, LAM1-116 staining of wild type and LΔCyto transfectants. Histograms are representative of 2 separate experiments. Data was generated by Dr. Doug Steeber at Duke University.
Figure 3.8. LAM1-116 treatment of wild type and cytoplasmic tail deletion transfectants upregulates the EL-246 epitope. Panel A, EL-246 staining on wild type transfectants (thin line), EL-246 staining with LAM1-116 treatment (thick line), and second stage goat anti-mouse FITC alone (dotted line). L-selectin staining by LAM1-116 is shown to the immediate left. Panel B, EL-246 staining on cytoplasmic tail deletion transfectants (thin line), EL-246 staining with LAM1-116 treatment (thick line), and second stage goat anti-mouse FITC alone (dotted line). L-selectin staining by LAM1-116 is shown to the immediate left. Histograms are representative of 2 separate experiments and were performed by Dr. Doug Steeber at Duke University Medical Center.
and B). There was a dramatic upregulation of the EL-246 epitope on the cytoplasmic tail mutants as the EL-246 epitope expression increased well beyond the minimal staining levels seen in Figure 3.7. These data suggest that an intact cytoplasmic tail of L-selectin is not need for the LAM1-116 induction of the conformational change that increases EL-246 epitope expression.

Discussion

In this report, we demonstrate a conformation-induced change in L-selectin mediated by a mAb that recognizes a functionally conserved epitope on L-selectin. The mAb, LAM1-116, generated in L-selectin deficient mice, specifically caused a conformational change in L-selectin as measured by an increase in FITC-labeled EL-246 staining of L-selectin (Figure 3.1). This effect was specific to mAb LAM1-116. Nineteen other anti-L-selectin mAbs did not induce the EL-246 epitope, nor did other antibodies against cell surface markers, such as the CD18 integrins and CD44 (Figure 3.2A and data not shown). Also, LAM1-116 induction of the EL-246 epitope was specific to the EL-246 mAb. Other mAbs against L-selectin, as well as mAbs directed against other cell surface markers, were not enhanced by treatment with LAM1-116 (data not shown and Figure 3.2B). These data relating to the specificity of this interaction are important since others have shown that L-selectin engagement by some mAbs can activate cellular processes and cause integrin-dependent adhesion of neutrophils under flow (Steeber et al., 1997, and Gopalan et al., 1997).
As stated previously, EL-246 recognizes a conserved epitope on both L-selectin and E-selectin (Jutila et al., 1992). Past mapping studies using L-selectin/P-selectin chimeras showed that the EL-246 epitope requires the SCR domains, as well as the lectin domain, for optimal antibody binding (Jutila et al., 1992). At that time, it was proposed that the EL-246 epitope was contained within the L-selectin SCR domains. These studies were extended here and we now show that none of the domains of L-selectin can, by themselves, confer the EL-246 epitope. Staining of a number of recombinant selectins containing different domains of L-, E-, and P-selectin show that optimal presentation of the EL-246 epitope in the absence of LAM1-116 requires both the lectin and SCR domains from E- or L-selectin and a complete cytoplasmic tail (Figure 3.7 and Table 3.2).

Recently, studies have been done which analyze L-selectin function inside the cell. Studies done by Pavalko et al., (1995) first described a linkage between the cytoplasmic tail of L-selectin and cytoskeletal proteins, and this observation was shown to be functionally important in intact cells by Evans and colleagues (1999). The latter was the first report of dynamic cytoskeletal association of L-selectin with the actin cytoskeleton, presumably by a linkage of α-actinin with the cytoplasmic tail of L-selectin and the actin cytoskeleton. Our studies show that the conformation-induced change in L-selectin, caused by LAM1-116, predisposes the protein to cytoskeletal association, which is triggered by EL-246 (Figures 3.5 and 3.6). Other mAbs directed against functionally distinct epitopes failed to induce cytoskeletal association of L-selectin (data not shown).
Thus, we have expanded on the data presented by Evans and colleagues (1999) and demonstrate that a specific conformation of L-selectin predisposes the protein to cytoskeletal association in the absence of crosslinking (Figures 3.5 and 3.6). We propose that this conformational change regulates L-selectin such that a high affinity binding epitope, the EL-246 epitope, is increased in expression and when this epitope is engaged, dynamic cytoskeletal association takes place. This data parallels other reports of conformation playing a role in L-selectin function. Studies on L-selectin shedding have shown that the cleavage site recognized by the membrane-bound metalloprotease has relaxed sequence specificity suggesting that the conformation of L-selectin is the most important factor in protease recognition and this conformation may be regulated by the presence of calmodulin binding to the cytoplasmic tail (Chen et al., 1995, Migaki et al., 1995, and Kahn et al., 1998).

As stated earlier, others have shown that L-selectin can act as a signal transduction molecule (Po et al., 1995, Waddell et al., 1995, Crockett-Torabi et al., 1995, Simon et al., 1995, Sikorski et al., 1996, Gopalan et al., 1997, and Steeber et al., 1997). However, a signaling event through L-selectin is not responsible for the increased binding of EL-246 to L-selectin. The event occurs at 4°C and in the presence of sodium azide and tyrosine kinase inhibitors (Table 3.1). Furthermore, LAM1-116 induces the EL-246 epitope on L-selectin in detergent lysates. This latter finding is important, since EL-246 does not recognize shed, soluble L-selectin very well and is, generally, a poor immunoprecipitating
antibody (M.A. Jutila, unpublished observation). A previous report by Schleiffenbaum and colleagues (1992) has shown that soluble L-selectin is conformationally distinct from cell surface L-selectin which may be the reason for poor L-selectin immunoprecipitation by EL-246, although EL-246 binding was not tested in their study. LAM1-116 likely alters this distinct conformation of soluble L-selectin. These observations clearly show that the cell is not needed for the enhancement of EL-246 epitope and a cellular signal is not required for increased presentation of the EL-246 epitope. Thus, if our hypothesis is correct, overt cellular signaling is not needed for increased functional activity of L-selectin.

The cytoplasmic tail of L-selectin is important for adhesive events including leukocyte rolling, receptor avidity, and cytoskeletal association of L-selectin (Kansas et al., 1993, and Evans et al., 1999). Thus, we tested whether or not the expression of specific epitopes on L-selectin are regulated by the cytoplasmic tail. In comparison to wild type L-selectin transfectant staining, the EL-246 staining of LΔCyto L-selectin was reduced to minimal levels (Figure 3.7A). In contrast, staining of the LAM1-116 epitope on the LΔCyto transfectants remained high (Figure 3.7B). However, in the presence of LAM1-116, the EL-246 epitope can be enhanced on the LΔCyto transfectants (Figure 3.8) suggesting that an intact cytoplasmic tail is not required for the conformational change induced in L-selectin and the subsequent increase in the El-246 epitope expression.

Based on our data, we propose that initial engagement with ligand induces a conformational change in L-selectin, leading to exposure of a high avidity binding site (EL-
246 epitope) for the original ligand, or possibly a second ligand, to bind and induce stronger tethers and slower rolling. In effect, our data also suggests that LAM1-116 binding to L-selectin may make it more “E-selectin-like.” E-selectin is an inducible member of the selectin family of adhesion molecules and is expressed on the cell surface at its highest levels 4-6 hours after an activation signal, such as TNF-α. It is synthesized de novo after activation and is expressed in a functional form that allows immediate capture of leukocytes from the blood stream. After the primary activation signal on the endothelium, no further activation is needed to mediate E-selectin capture of leukocytes. E-selectin mediates a more avid interaction than L-selectin, since leukocyte rolling is slower on the former in assays done under similar shear forces (Alon et al., 1997). In vivo, L- and E-selectin can recognize the same naturally occurring ligands on HEV (Mebius and Watson, 1993, and Tu et al., 1999). Past studies in our lab have demonstrated that moving E-selectin-expressing cells to 37°C does not decrease EL-246 staining. In addition, EL-246 preferentially binds to E-selectin versus L-selectin in competitive binding assays (Bargatze et al., 1994b). If this model is correct, it could explain why EL-246 if effective as a therapeutic agent during ischemia and reperfusion injury, as well as other shear dependent events (Ma et al., 1993, Steinberg et al., 1994, Pizcueta and Luscinskas, 1994, Ramamoorthy et al., 1996, Seekamp et al., 1997, and Stotland and Kerrigan, 1997). Presently, we are not able to functionally test the hypothesis that LAM1-116 induces higher ligand binding avidity, because both mAbs
(LAM1-116 and EL-246) and native ligand, block the function of L-selectin. However, studies are under way comparing binding affinities of EL-246 for E-selectin and LAM1-116-bound L-selectin.

Here, we report that a conformational change in L-selectin induced by an artificial ligand may play an important regulatory role in the molecular interaction of L-selectin with its ligands, and predisposes the protein to cytoskeletal association upon engagement of a second functionally important epitope. The functional importance of the conformational change described is currently under investigation. Our report provides additional insight into the structural features of L-selectin and may lead to a better understanding of L-selectin/ligand interactions.
References Cited


CHAPTER 4

COMPARISON OF L-SELECTIN CYTOSKELETAL ASSOCIATION TO THAT OF OTHER HUMAN AND BOVINE LYMPHOCYTE SURFACE ANTIGENS

Introduction

Lymphocyte trafficking and recirculation is essential for maintaining a healthy immune system. Various adhesion molecules on the leukocyte surface, including L-selectin, are involved in trafficking and mediate interactions with the vascular endothelium as well as with other immobilized leukocytes (Butcher, 1999, Bargatze et al., 1994, and Jutila and Kurk, 1996). L-selectin is constitutively expressed on leukocytes and is involved in tethering and rolling as it engages appropriate carbohydrate ligands expressed on the endothelium or other leukocytes. The interactions of L-selectin and its ligands mediate specific trafficking events in vivo. For example, L-selectin binds to the peripheral node addressin, a group of glycoproteins collectively termed PNAd, and this interaction controls lymphocyte trafficking into peripheral lymph nodes (Gallatin et al., 1983, Streeter et al., 1988, Lasky et al., 1992, Berg et al., 1991, Hemmerich et al., 1994, and Sassetti et al., 1998, Kansas, 1996, von Andrian et al., 1996, and Butcher, 1999). When PNAd is expressed on the mucosal addressin, MAdCAM-1, L-selectin can also mediate lymphocyte traffic into intestinal sites (Berg et al., 1993, and Butcher, 1999). Leukocyte-leukocyte rolling, controlled by an L-selectin/P-selectin glycoprotein ligand -1 interaction, also plays a role in trafficking by augmenting endothelial capture and rolling of other
leukocytes (Walcheck et al., 1996). In addition, cells that do not express L-selectin do not home efficiently to sites of inflammation (Jutila et al., 1989).

Recently, it has been shown that L-selectin dynamically associates with the actin cytoskeleton by mAb crosslinking, hyperthermic treatment, or ligand binding (Evans et al., 1999). We have demonstrated that upon induction of a conformational change in L-selectin and subsequent engagement of a conserved epitope on L-selectin, cytoskeletal association takes place in the absence of antibody crosslinking (Chapter 3). Presumably, L-selectin associates with the actin cytoskeleton by linkage of α-actinin to the cytoplasmic tail of L-selectin and the actin cytoskeleton (Pavalko et al., 1995 and Evans et al., 1999). Previous work has demonstrated that the cytoplasmic tail of L-selectin is important to L-selectin function as mutants lacking the 11 terminal amino acids of the cytoplasmic tail do not exhibit rolling interactions in vitro and in vivo (Kansas et al., 1993). These same cells are also incapable of L-selectin cytoskeletal linkage under a variety of conditions (Evans et al., 1999).

Cytoskeletal association of adhesion molecules has been investigated for some time. Most studies have focused on integrin function as it relates to the cytoskeleton controlling the avidity of β1 and β2 integrins in leukocytes (Shaw et al., 1990, Pavalko and LaRoche, 1993, Otey et al., 1993, Pardi et al., 1992, Pavalko and Otey, 1994, and Sampath et al., 1998). These reports suggest that integrins associate with detergent resistant membranes (DRMs) by direct binding of the integrin cytoplasmic tail to the
actin cytoskeleton. A variety of other proteins have been studied as well, including CD2, CD3, CD4, CD5, CD8, CD9, CD28, CD44, CD45, and class I MHC (Geppert and Lipsky, 1991 Gur et al., 1997, and Yashiro-Ohtani et al., 2000). Depending upon the treatment of specific proteins by either primary antibody alone or by antibody crosslinking, these proteins can be induced to associate with the cytoskeleton. In addition, numerous signaling molecules are thought to constitutively associate with the cytoskeleton through GPI-anchored interactions that form what some refer to as “lipid rafts” and play a role in T cell activation (Brown and Rose, 1992, Moran and Miceli, 1998, and Penninger and Crabtree, 1999). However, the term lipid rafts has since been broadened to other interactions such as the TCR and BCR (Janes et al., 1999, and Cheng et al., 1999). Further studies have shown that supramolecular activation clusters (SMACs) exist as organized contact sites at the physical interface between T cells and antigen presenting cells and the proteins involved at such sites are linked to the cytoskeleton (Monks et al., 1997, Shaw and Dustin, 1997, and Monks et al., 1998). Recent studies have also elucidated the importance of the Ras superfamily of small guanosine triphosphates (GTPases), especially the Rho family, and the actin cytoskeleton, in immune cell function (reviewed in Hall, 1998, and Magee and Marshall, 1999). Indeed, Rho family deficiencies, in which cytoskeletal association is impaired, result in abnormalities in neutrophil function and host defense, decreased integrin-mediated cell adhesion in a lymphoid cell line, decreased cell motility in neutrophils and

Cell membranes are composed of a variety of compounds, including saturated fatty acids, and their alteration can disrupt membrane linkage to the cytoskeleton. For example, polyunsaturated fatty acids (PUFAs) can replace the saturated fatty acids in the cell membrane when taken through diet or incubated directly with cultured cells (Li and Steiner, 1991, Yosefy et al., 1996, Khalfoun et al., 1997, and Stulnig et al., 1998). PUFA treatment of immune cells has a wide range of effects that are both immunoregulatory and anti-inflammatory (Calder, 1998). The mechanism of PUFA action is not clear but is hypothesized to act via effects on cell adhesion molecules, the cytoskeleton, PKC, eicosanoid receptors, prostaglandin synthases, and/or lipoxygenases (Chen et al., 1992, and Johanning and Lin, 1995). However, a recent report demonstrates that enrichment of the T cell membrane with PUFAs disrupts the ability of T cells to signal via the TCR by disrupting the cytoskeletal association of Lck and Fyn kinases with the cytoskeleton (Stulnig et al., 1998).

To further elucidate the nature of L-selectin cytoskeletal association, we have examined a variety of cell surface proteins on human and bovine lymphocytes and compared their patterns of association to that of L-selectin. Our data provides a comprehensive analysis of the lymphocyte cytoskeleton at the ultrastructural level and describes three cytoskeletal linkage patterns of various proteins in relation to L-selectin.
We also analyze the impact of PUFA treatment on the cytoskeletal association of L-selectin.  

**Materials and Methods**

**Animals**

One-week-to-three-month-old Holstein calves, housed in the Montana State University large animal facility, and healthy human donors were used as sources of peripheral blood.

**Monoclonal Antibodies**

The following mAbs were used. Anti-L-selectin included DREG 55, DREG 56, DREG 200 (Kishimoto et al., 1990), LAM1-116 (Steeber et al., 1997), GD 4.22 (Jutila and Kurk, 1996), and EL-246 (Jutila et al., 1992). Anti-human αβ and γδ T cell receptor included WT-31 and γδ1, respectively (Becton Dickenson, Mountain View, CA). Anti-bovine γδ T cell receptor included GD 3.8, GD 3.1, and GD 197 (Wilson et al., 1999), and anti-WC1 included IL-A29 (American Tissue Type Culture Collection, Manassas, VA) and GD 6.22a (deBoer and Jutila, unpublished). Anti-CD2 included CC42 for bovine studies (Naessens, et al., 1997) and T11-Fluorescein Isothiocyanate (FITC) on human cells (Coulter Immunology, Hialeah, FL). Anti-CD3 included MM1A on bovine cells (VMRD, Pullman, WA) and CD3-FITC for human studies (Becton Dickenson). Anti-CD4 included CC30 on bovine cells (Naessens et al., 1997) and Leu-3-FITC on human
cells (Becton Dickenson), and anti-CD5 included CC17 on bovine cells (Naessens et al., 1997). Anti-CD8 included CC58 on bovine cells (Naessens et al., 1997) and Leu-2a-Phycoerythrin (PE) on human cells (Becton Dickenson). Anti-CD18 included IB4 and MHM-23 for bovine studies (ATCC #10164, and Dako, Carpinteria, CA, respectively) and MHM-23 and RDI 68-5A5 for human studies (Dako and Research Diagnostics Inc., Flanders NJ, respectively) and anti-CD44 included Hermes-3 (Picker et al., 1989). Anti-CD45 RA included GS5A on bovine cells (VMRD) and Leu-16 on human cells (Becton Dickenson). Anti-CD45 RO included GD 6.10a on bovine cells (Walcheck, de Boer and Jutila, unpublished) and CD45 RO for human studies (Becton Dickenson). Anti-B-cell included CD19 on human cells (Becton Dickenson), and anti-E-selectin ligand(s) included P11.4 chimera/human IgG (Tsang, et al., 1995, and Jones et al., 1997). Anti-GD 3.5, an unknown antigen on bovine γδ T cells, included mAb GD 3.5 (Jones et al., 1996), and BN1-80, an unknown antigen on bovine monocytes and a small fraction of neutrophils, included BN1-80 (Soltys et al., 1999). FITC- or PE-conjugated goat anti-mouse secondary antibody was added to samples for visualization by flow cytometry where appropriate (Jackson ImmunoResearch, West Grove, PA). For visualization of the P11.4 chimera, R-PE-conjugated F(ab')2 goat anti-human IgG, Fcγ specific, was added (Jackson).
Cell Preparations

Peripheral blood was collected by venipuncture into citrate or heparin anticoagulant tubes as described previously (Jones et al., 1997). Whole blood was diluted 1:1 with Hanks' balanced salt solution (HBSS, Gibco, BRL, Grand Island, NY), underlayed with Histopaque 1077 (Sigma, St. Louis, MO) and centrifuged at 700 g for 30 minutes. The buffy coat containing peripheral blood mononuclear cells was collected, washed with HBSS, and if red blood cells were present, they were lysed in a hypotonic solution for 10 seconds followed by rapid dilution in HBSS and centrifuged. In some cases, monocytes were depleted from the sample by incubation on plastic in Dulbecco's Modified Eagle Medium (DMEM, Sigma) containing 0.7% bovine serum albumin (BSA, Sigma) for 30 mins at 37°C. Purified lymphocytes were collected, centrifuged, and suspended in either HBSS or phosphate buffered saline (PBS, Sigma).

Scanning and Transmission Electron Microscopy

Bovine γδ T cells were collected and prepared for scanning electron microscopy, as described (Leid et al., 1998). Briefly, γδ T cells were treated with Nonidet-40 detergent lysis buffer (150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Hepes, 2% goat serum, and 0.5% NP-40) (cytoskeletal buffer) as described (Nebe et al., 1997) for 15 min at room temperature (RT), or not, washed in PBS containing 0.7% horse serum, and adhered to 12 mm glass cover slips (Ted Pella, Redding CA) coated with CellTak® (100 μg/mL, Collaborative Biomedical Products). Adherent cells were fixed in 2.5% (v/v)
glutaraldehyde in Millonig's phosphate buffer overnight, dehydrated, critical point dried, sputter coated with gold-palladium, and examined with a JEOL 100CX scanning/transmission electron microscope (Speer et al., 1979).

Transmission electron microscopy (TEM) studies were done as described (Leid et al., 1998). Briefly, bovine lymphocytes were isolated as above, treated with cytoskeletal buffer for 15 min at RT, or not, washed, and fixed in modified Karnovsky's fixative overnight. Samples were partially dehydrated in ethanol, prestained with 1% (w/v) phosphotungstic acid (Ted Pella) and 1% (w/v) uranyl acetate (Ted Pella) in 70% ethanol, completely dehydrated and embedded in Spurr's (Ted Pella) medium. Ultrathin sections were stained with lead citrate and examined by TEM.

Flow Cytometric Analysis of Non-detergent-treated Cells

Flow cytometry was performed as previously described (Leid et al., 1998). Briefly, lymphocytes were collected, suspended at 1x10^7 cells/mL in HBSS, and 100 μL aliquots transferred into FACS tubes (Fisher Scientific Co., Pittsburgh, PA) for staining. The mAbs described above were added to the cells, incubated on ice for 30 min, washed in PBS containing horse serum and sodium azide (FACS buffer), and, if appropriate, fluorochrome-labeled second stage was added and the cells incubated for an additional 30 min on ice. The cells were washed a final time, resuspended in FACS buffer, and analyzed on either a FACScan or FASCaliber (Becton Dickenson). Second stage
fluorochrome alone was added as a control for background staining. For each sample, 10,000-20,000 events were collected and representative histograms are shown.

Flow Cytometric Analysis of Detergent-Treated Cells

Cells membrane protein linkage to the cytoskeleton was assayed by flow cytometry as described with some exceptions (Geppert and Lipsky, 1991 and Nebe et al., 1997). In order to more thoroughly study protein linkage to the cytoskeleton, three separate detergent treatments were developed and we have classified each type of association as either constitutive, inductive, or mAb crosslink-induced. **Constitutive:** lymphocytes were treated first with 100 μL of cytoskeletal buffer for 15 min at RT, washed with FACS buffer, and primary mAb added and incubated on ice for 30 min. These samples were then washed again and, if appropriate, second stage FITC or PE-conjugated goat anti-mouse antibodies added, incubated for 30 min on ice, washed a final time, and analyzed by flow cytometry. Staining only occurred for those proteins linked to the cytoskeleton prior to detergent treatment. **Inductive:** lymphocytes were treated with primary mAb for 15 min at 37°C, washed, and 100 μL of cytoskeletal buffer added to each sample for 15 min at RT. Samples were washed, second stage fluorochrome-labeled antibodies added if necessary for 30 min on ice, washed a final time, and analyzed by flow cytometry. This treatment procedure allowed for visualization of mAb or chimera/Ig induced association to the cytoskeleton in the absence of secondary mAb-
induced crosslinking. **MAb Crosslink-induced:** lymphocytes were treated with primary mAb for 15 min at 37°C, washed, and either PE- or FITC-labeled second stage antibodies added to the appropriate samples for 30 min on ice to induce crosslinking. In the event that directly fluorochrome-labeled antibody was utilized in this treatment procedure, unlabeled goat anti-mouse IgG was added as a crosslinking agent, as described (Geppert and Lipsky, 1991). The cells were then washed, treated with 100 μL of cytoskeletal buffer for 15 min at RT, washed again, and analyzed by flow cytometry. Mock cytoskeletal buffer without NP-40 was used as a control for all procedures and did not affect protein expression (data not shown). Second stage FITC- or PE-labeled goat anti-mouse and anti-human antibody alone was added as a control for background staining in all three treatments. Staining was measured by gating on the detergent insoluble cytoskeletal fraction (see Figure 4.1) by forward and side scatter. The gated population was confirmed to be the cytoskeleton by phalloidin-FITC (Sigma), which specifically stains the detergent insoluble cytoskeleton (Nebe et al., 1997). For each experiment, 10,000-20,000 events were collected.

**SDS-PAGE Analysis of Detergent-soluble and Detergent-insoluble Membrane Fractions**

Human and bovine lymphocytes were surfaced labeled with biotin, as described (Jones et al., 1997), and low dose detergent soluble and insoluble membrane fractions prepared according to the following procedure. Cell preparations were treated with
cytoskeletal buffer containing 0.5% NP-40 for 15 min at RT, centrifuged for 5 min at 200 g, and the supernatant fluid collected. This aliquot was labeled the detergent-soluble membrane fraction. The detergent-insoluble pellet was further treated with 2% NP-40 buffer for 1 hr on ice with constant vortexing, centrifuged at 200g, and the supernatant fluid collected. This aliquot was labeled the detergent-insoluble membrane fraction. Each preparation was then incubated with mouse serum and protein G beads (Boehringer Mannheim, Germany) overnight at 4°C under constant rotation to reduce non-specific immunoprecipitation as described (Jones et al., 1996). The samples were centrifuged, the supernatant fluid collected, and the beads discarded. Samples were aliquoted into 120 μL fractions and 10 μg of mAb added at RT for 1 hour, and proteins immunoprecipitated overnight with protein G at 4°C under constant rotation. The beads were washed 3X with wash buffer, mixed with non-reducing buffer, boiled for 3 min, centrifuged, and loaded directly onto an 8% polyacrylamide gel. Gels were electrophoresed and the proteins were transferred to PVDF (Bio-Rad, Hercules, CA) overnight at 4°C. Proteins were visualized using a streptavidin horseradish peroxidase (Amersham, Buckinghamshire, England) reaction and ECL (Amersham) detection system and developed on X-OMAT (Kodak) film.
PUFA Enrichment of Bovine T Cells

Fatty acid composition of T cell membranes was modified as described (Stulnig et al., 1998). Briefly, bovine lymphocytes were incubated with 25-50 μM of PUFAs (Cayman Chemical Co., MI) for 2 days in DMEM containing penicillin/streptomycin (50 U/mL and 50 μg/mL, respectively, Gibco BRL, Gaithersburg, MD), 2 mM glutamine (Gibco BRL), 0.4% (w/v) BSA (fraction V, containing less than 3 μM total fatty acids, Sigma), 1 mg/L transferrin, and 8.1 mg/L α-monothioglycerol at 37°C in humidified atmosphere in the presence of 10% CO₂. Control cells were incubated in DMEM containing 10% BSA, and glutamine and antibiotics as above. Cell viability after culture was >90% as determined by trypan blue exclusion. PUFA-cultured and control cultured cells were prepared for FACS and analyzed as described above.

Results

Detergent Treatment of Human and Bovine Lymphocytes Generated a Homogeneous Population of Actin Cytoskeleton Particles

Previous reports have demonstrated that protein association with the actin cytoskeleton can be studied by flow cytometry (Nebe et al., 1997, Gur, et al., 1997, and Capote and Maccioni, 1998). One characteristic of the actin cytoskeletal network is that phalloidin, a phallotoxin, specifically stains F-actin and can be used to positively identify
Figure 4.1. Phalloidin-FITC specifically stains detergent-treated cytoskeletal particles versus intact, non-detergent treated cells. Panel A, scatter profile of cytoskeletal particles showing uniform, small size. Panel B, staining of the particles in Panel A with phalloidin-FITC. Panel C, scatter profile of non-detergent-treated bovine peripheral blood mononuclear cells (PBMCs) with a selective gate around the lymphocyte population. Panel D, staining of the gated population of PBMCs with phalloidin-FITC. Note the lack of fluorescence of non-detergent treated cells by phalloidin-FITC staining. Data are representative of 3 separate experiments.
cytoskeletal particles by flow cytometry. Low dose detergent treatment of human or bovine lymphocytes generated a homogeneous population of cytoskeletal particles relatively small in size which were stained brightly by phalloidin-FITC (Figure 4.1, A and B). In contrast, non-detergent-treated cells maintained their normal size and were not stained by phalloidin-FITC (Figure 4.1, C and D).

Electron Microscopy Studies on the Actin Cytoskeleton

In an effort to further understand the nature of the lymphocyte cytoskeleton and the particles analyzed in Figure 4.1, scanning and transmission electron microscopy techniques were employed. By scanning electron microscopy (SEM), the surface of the actin cytoskeleton was smooth, with little or no surface topology compared to that of an intact, non-detergent-treated cell (Figure 4.2A, intact bovine γδ T cell, and B, bovine γδ T cell cytoskeleton). Note the numerous microvilli on the non-detergent-treated, intact γδ T cell versus the detergent-treated γδ T cell, and the smaller size of the detergent-treated γδ T cell compared to a non-detergent-treated γδ T cell (3 μm versus 4-8 μm for bovine and human lymphocytes, Leid et al., 1998, and Picker et al., 1991).

Transmission electron microscopy was utilized to investigate the internal makeup of the cytoskeletal sphere seen in Figure 4.2. Strikingly, detergent treatment of bovine γδ T cells left highly uniform cellular ‘ghosts’ that consisted primarily of the cell nucleus,
Figure 4.2. Scanning electron micrographs of an untreated and a detergent-treated bovine γδ T cell. Panel A, untreated γδ T cell showing numerous microvilli (Mv). Original magnification, X15,000. Panel B, cellular remnant of a low dose detergent-treated bovine γδ T cell showing a smooth surface with little or no surface topography. Original magnification, X20,000.
Figure 4.3. Transmission electron micrographs of untreated and detergent-treated bovine lymphocytes. Panel A, untreated γδ T cell showing numerous microvilli (Mv), euchromatin (Eu), Golgi complex (Go), heterochromatin (Hc), mitochondria (Mi), nuclear envelope (Ne), and plasmalemma (Pl). Original magnification, X25,000. Panel B, bovine lymphocyte treated with low dose detergent showing incomplete plasmalemma (Pl), indistinct nuclear envelope (Ne), and lack of cytoplasmic organelles; euchromatin (Eu); heterochromatin (Hc); and nucleolus (No). Original magnification, X48,000.
and had little cytoplasm and few or no cellular organelles (Figure 4.3B). The TEM of the detergent-treated lymphocytes showed that the smooth surface seen by SEM was actually the plasmalemma of the cell. By TEM, the plasmalemma was found to have collapsed upon and surrounded the nucleus. This was in contrast to a non-detergent-treated bovine lymphocyte, which had an intact plasmalemma, nuclear membrane, cytoplasm and typical cytoplasmic organelles (Figure 4.3A).

Flow Cytometric Analysis of Human and Bovine Lymphocyte Cytoskeletal Association of Cell Surface Proteins

L-selectin has previously been shown to dynamically associate with the actin cytoskeleton (Evans et al., 1999). Therefore, we wanted to compare the association of numerous proteins on the surface of human and bovine lymphocytes in an effort to relate patterns of association to their known functions. Human lymphocytes were treated as described in Materials and Methods and analyzed by flow cytometry. As previously described (Geppert and Lipsky, 1991), CD44 was found to constitutively associate with the actin cytoskeleton following detergent treatment, and exhibited bright staining patterns under all three treatment conditions (Figure 4.4A). CD3, a co-stimulatory molecule, could be induced to associate with the cytoskeleton by primary mAb binding but was absent if lymphocytes were treated with detergent prior to mAb binding alone (Figure 4.4B). Similar results were seen with a FITC conjugated anti-CD3 mAb (data not shown). In contrast, the αβ TCR on human lymphocytes was only linked after
Figure 4.4. Flow cytometric analysis of the cytoskeletal association of human lymphocyte surface proteins. Panel A, CD44 is constitutively associated with the cytoskeleton (thick line, constitutive*; thin line, inductive*; dotted line, mAb crosslink*). Panel B, CD3 is induced to associate with the cytoskeleton by mAb binding alone (constitutive*, inductive*, mAb crosslink*). Panel C, αβ TcR links to the cytoskeleton only when crosslinked by secondary mAb (constitutive*, inductive*, mAb crosslink*). Control stains of non-detergent-treated cells are shown to the right of each cytoskeletal histogram (thick line, mAb staining; dotted line, second stage goat anti-mouse F(ab')2 labeled fluorochrome. Data are representative of 3 separate experiments.

*See Materials and Methods for details on treatments.
crosslinking with a secondary antibody, similar to L-selectin (Figures 4.4C, and Evans et al., 1999). Control staining of non-detergent-treated protein expression is shown to the immediate right of each histogram (Figure 4).

WC1, a lineage specific antigen on the surface of bovine γδ T cells, was found to constitutively associate with the actin cytoskeleton (Figure 4.5A). This result was similar to that seen for CD44 and other proteins examined (Figure 4.4, and data not shown), although treatment with primary mAb alone, as well as crosslinking, increased the amount of WC1 associated with the cytoskeleton compared to CD44. Another mAb that recognizes WC1, GD 6.22a, produced similar results (data not shown). Bovine γδ TCR, like human CD3 above, could be induced to associate with the cytoskeleton by primary mAb binding alone (Figure 4.5B). Directly FITC-conjugated mAb gave similar results (data not shown). This result was in contrast to the human αβ TCR seen above. Three unique bovine anti-γδ TCR mAbs all induced cytoskeletal association, suggesting the effect was not due to engagement of a specific epitope on the γδ TCR (data not shown and Figure 4.7). Finally, as previously shown, L-selectin did not link to the cytoskeleton until crosslinked by secondary antibodies (Figure 4.5C). Control staining of non-detergent-treated protein expression is shown immediately to the right of each histogram.
Figure 4.5. Flow cytometric analysis of the cytoskeletal association of bovine lymphocyte surface proteins. Panel A, WC1 is constitutively associated with the cytoskeleton (thick line, constitutive*; thin line, inductive*; dotted line, crosslink*). Panel B, γδ TCR is induced to associate with the cytoskeleton by mAb binding alone (constitutive*, inductive*, mAb crosslink*). Panel C, L-selectin links to the cytoskeleton only when crosslinked by secondary mAb (constitutive*, inductive*, mAb crosslink*). Control stains of non-detergent-treated cells are shown to the right of each cytoskeletal histogram (thick line, mAb staining; dotted line, second stage goat anti-mouse F(ab')2 labeled fluorochrome. Data are representative of 3 separate experiments.

*See Materials and Methods for details on treatments.
SDS-PAGE Analysis of Detergent-soluble and Detergent-insoluble Membrane Fractions

In order to confirm our flow cytometry results at the protein level, SDS-PAGE analysis of immunoprecipitated proteins was performed. Membrane fractions were prepared as in Materials and Methods and antigens immunoprecipitated with mAbs. CD44 was found in the detergent-insoluble membrane fraction, parallel to the results seen with flow cytometric studies (Figure 4.6, lane 5). CD44 was also found in the detergent-soluble fraction, indicating that either some CD44 was not constitutively linked to the cytoskeleton or that upon mild detergent treatment, some CD44 was lost from the surface (Figure 4.6, lane 6). As mentioned above, CD18 was consistently found to be weakly associated with the detergent-insoluble membrane fraction (Figure 4.6, lane 7). However, it was readily precipitated from the detergent-soluble fraction (Figure 4.6, lanes 7). In contrast, L-selectin and E-selectin ligand(s) were not seen in the insoluble fraction, in support of the flow data (Figure 4.6, lane 1 versus 2, and 3 versus 4, respectively). The arrow points to a specific E-selectin ligand band at 130 kD.

SDS-PAGE analysis of bovine lymphocyte surface antigens also confirmed the flow cytometry data. WCI, a lineage specific γ&delta; T cell marker thought to function in various capacities as a signal transduction protein, and CD5, an important protein involved in T and B cell differentiation and function, both were immunoprecipitated in the detergent-insoluble membrane fraction (Figure 4.7, lanes 1 and 3, respectively). Similar to the human studies with CD44, both proteins were also present in the detergent-soluble
Figure 4.6. SDS-PAGE analysis of human lymphocyte surface proteins in detergent-soluble (S) and detergent-insoluble (IN) membrane fractions. Lanes 1 and 2, L-selectin; lanes 3 and 4, P11.4 chimera; lanes 5 and 6, CD44; and lanes 7 and 8, CD18. Single arrow denotes specific E-selectin ligand immunoprecipitation band ~130 kD. Double arrows denote specific CD18 immunoprecipitation bands. Note weak presence in the detergent-insoluble membrane fraction. Data are representative of 3 separate experiments.

fraction (Figure 4.7, lanes 2 and 4, respectively). CD4, which was not seen constitutively associated with the cytoskeleton by FACS, was weakly associated by SDS-PAGE analysis (Figure 4.7, lane 5). However, most CD4 was seen in the detergent-soluble membrane fraction (Figure 4.7, lane 6). Bovine CD3 and CD2 were found only in the detergent-soluble membrane fraction (Figure 4.7, lanes 8 and 10 respectively). As seen in the human studies above, L-selectin was predominantly found in the detergent-soluble
membrane fraction (Figure 4.7, lane 12), though a small amount was precipitated from the detergent-insoluble membrane fraction (Figure 4.7, lane 11). Previous reports have suggested that a small amount of L-selectin may be constitutively associated with the cytoskeleton (Pavalko et al., 1995, and Evans et al., 1999), thus explaining our immunoprecipitation results. However, it is clear, as these papers suggest, that most L-selectin is not constitutively associated with the actin-based cytoskeleton (Figure 4.7, lane 11 versus 12). E-selectin ligand(s) were not precipitated from the detergent-insoluble membrane fraction in the absence of E-selectin chimera (Figure 7, lanes 13 versus 14). The arrow denotes a specific band that has been shown previously to be an E-selectin ligand on bovine lymphocytes (Jones et al., 1997). As mentioned above, the bovine γδ TCR was not constitutively linked to the cytoskeleton, but three distinct bovine anti-γδ TCR mAbs alone could induce the TCR to associate with the cytoskeleton (Figure 4.5). Accordingly, bovine γδ TCR was specifically immunoprecipitated from the detergent-soluble membrane fraction by anti-γδ TCR mAbs GD 197, and GD 3.1 (Figure 4.7, lanes 16 and 18, respectively). Thus, in addition to mAb GD 3.8 (Figure 4.5B, and data not shown), GD 197 and GD 3.1 also demonstrate that bovine γδ TCR is not constitutively associated with the cytoskeleton.
Characterization of Other Human and Bovine Surface Proteins

Further characterization of additional human and bovine cell membrane proteins was undertaken as well, and those results are summarized in Tables 4.1 and 4.2. Based upon data generated by flow cytometry, all the proteins were grouped into 3 patterns of cytoskeletal association: constitutive, inductive, and mAb crosslink-induced.
Table 4.1. Human Lymphocyte Cytoskeletal Association of Various Proteins

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Constitutive</th>
<th>Inductive</th>
<th>Crosslink</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>CD3</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD4</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD8</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD18</td>
<td>Weak</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD44</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD45RA</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD45RO</td>
<td>No</td>
<td>Weak</td>
<td>Yes</td>
</tr>
<tr>
<td>αβ TCR</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>γδ TCR</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>E-selectin Ligand(s)</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>L-selectin*</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Five different anti-L-selectin mAbs were employed for these studies and all gave similar results (See Materials and Methods).

All the data in the table were generated from flow cytometric analysis with at least 3 separate experiments for each antigen. In some cases, SDS-PAGE was employed to confirm the flow cytometry data (data not shown).

PUFA Treatment Specifically Reduced L-selectin Expression and Inhibits L-selectin Cytoskeletal Association

PUFAs have previously been shown to decrease the expression level of some proteins, including E-selectin, as well as disrupt the ability of some proteins, such as Lck and Fyn, to associate with the cytoskeleton (Hoover et al., 1981, Stulnig et al., 1998, and De Caterina and Libby, 1996). We tested the effects of PUFA treatment on the linkage of representative surface antigens from each pattern of cytoskeletal association, which were L-selectin, TCR, and WC1, as presented in Tables 4.1 and 4.2. Enrichment of bovine lymphocyte cell membranes with Eicosapentaenoic Acid (EPA) reduced the expression
Table 4.2. Bovine Lymphocyte Cytoskeletal Association of Various Proteins

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Constitutive</th>
<th>Inductive</th>
<th>Crosslink</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD3</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD4</td>
<td>No*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD8</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD18</td>
<td>Weak</td>
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</tr>
<tr>
<td>CD45RA</td>
<td>No</td>
<td>Yes</td>
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</tr>
<tr>
<td>CD45RO</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>WC1 (IL-A29)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>WC1 (GD 6.22a)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GD 3.5</td>
<td>No</td>
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<td>γδ TCR (GD 3.8)</td>
<td>No</td>
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<tr>
<td>γδ TCR (GD 3.1)</td>
<td>No</td>
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</tr>
<tr>
<td>γδ TCR (GD 197)</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
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<td>BN1-80</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>E-selectin Ligand(s)</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>L-selectin**</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
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</tbody>
</table>

*There was a weak constitutive association of CD4 with the detergent-insoluble membrane fraction by SDS-PAGE gel electrophoresis. However, this result was not seen on a consistent basis.

**Five different anti-L-selectin mAbs were employed for these studies and all gave similar results.

All the data in the table were generated from flow cytometric analysis with at least 3 separate experiments for each antigen. In some cases, SDS-PAGE was employed to confirm the flow cytometry data (data not shown).

level of L-selectin by anti-L-selectin mAb DREG 56 staining compared to control (Figure 4.8A, mode fluorescence intensity average (MFI) of 98 ± 9 S.E.M. vs 187 ± 17 S.E.M. for 3 separate experiments, p<0.01). Docosapentaenoic Acid (DPA) or Docosahexaneoic acid (DHA) treatment gave similar results (data not shown). Interestingly, the expression of a conserved epitope on L-selectin, recognized by mAb EL-246, was dramatically
Figure 4.8. Treatment of bovine lymphocytes with EPA reduced the expression of L-selectin but did not alter the expression of γδ TCR or WC1. Panel A, staining of L-selectin by mAb DREG 56 on control cultured (c.c.) cells (thick line) versus EPA-treated cells (thin line). Panel B, staining of L-selectin by mAb EL-246 on c.c. (thick line) versus EPA-treated cells (thin line). Panel C, staining of γδ TCR by mAb GD 3.8 on c.c (thick line) versus EPA-treated cells (thin line). Panel D, staining of WC1 by mAb IL-A29 on c.c. (thick line) versus EPA-treated cells (thin line). Data are representative of 3 separate experiments.
reduced compared to its expression on control cultured cells (Figure 4.8B, MFI of 22 ± 8 S.E.M. vs 107 ± 10 S.E.M. for 3 separate experiments, p< 0.01). This result was not due to the absence of L-selectin as DREG 56 mAb stained EPA treated cells. In contrast to L-selectin, EPA treatment of bovine lymphocytes did not change the surface expression of γδ TCR or WC1 (Figure 4.8 C and D, respectively).

We have previously characterized a mAb-induced (LAM1-116) conformational change in L-selectin that increased the expression of another conserved epitope (EL-246) and predisposed L-selectin to cytoskeletal association (Chapter 3). Thus, we tested whether or not LAM1-116 could restore the loss of the EL-246 epitope expression on EPA-treated bovine lymphocytes. Enhancement of the EL-246 epitope occurred on L-selectin for both the control cultured and EPA-treated lymphocytes (Figure 4.9A and B, respectively).

L-selectin on untreated, resting cells can be induced to associate with the cytoskeleton by the mAb-induced conformational change described above as well as by antibody crosslinking. However, L-selectin on EPA-treated lymphocytes did not associate with the cytoskeleton by treatment with LAM1-116 and EL-246 (Figure 4.10A), in spite of the fact that LAM1-116 binding to L-selectin on EPA-treated cells did enhance the expression of the EL-246 epitope. This result was not due to the lack of surface expression of L-selectin, as detected by DREG 56 staining (Figure 4.10A). In contrast, L-selectin on bovine lymphocytes cultured without EPA was able to link to the
Figure 4.9. Enhancement of the EL-246 epitope by mAb LAM1-116 occurred on control cultured as well as EPA-treated cells as measured by EL-246-FITC staining of L-selectin. Panel A, LAM1-116 treatment of control cultured cells enhanced the expression of the EL-246 epitope (LAM1-116-treated, thick line, EL-246 alone, thin line). Panel B, LAM1-116 treatment of EPA cultured cells enhanced the expression of the EL-246 epitope (LAM1-116-treated, thick line, EL-246 alone, thin line). Data are representative of 3 separate experiments.

cytoskeleton by LAM1-116/EL-246 mAb engagement (Figure 4.10A). L-selectin on freshly isolated, as well as control cultured, bovine lymphocytes linked to the cytoskeleton by DREG 56 mAb crosslinking (Figure 4.10B). However, DREG 56 mAb crosslinking of L-selectin did not induce cytoskeletal association of L-selectin on EPA-
treated cells (Figure 4.10B). The lack of DREG 56 mAb crosslinking-induced L-selectin cytoskeletal association was not due to the absence of L-selectin on EPA-treated cells (Figure 10B). Furthermore, bovine \( \gamma \delta \) TCR and WC1 antigen were associated with the cytoskeleton on EPA-treated cells suggesting that the cytoskeletal-linkage machinery for these proteins was still intact (Figure 4.11A and B, respectively). Thus, PUFA treatment appears to have a unique effect on the linkage of only some cell surface proteins.
Figure 4.11. Cytoskeletal association of bovine $\gamma\delta$ TCR and WC1 is not altered by EPA treatment. Panel A, bovine $\gamma\delta$ TCR was associated with the cytoskeleton after treatment with the mAb GD 3.8 (thin line, control cultured, thick line, EPA-treated, and dotted line, second stage goat anti-mouse control. Panel B, WC1, a lineage specific marker on bovine $\gamma\delta$ T cells, associated with the cytoskeleton after treatment with EPA (thin line, control cultured, thick line, EPA-treated, and dotted line, second stage goat anti-mouse control). Data are representative of 2 separate experiments.

Discussion

This report expands upon previous knowledge of the lymphocyte cytoskeleton and provides additional insight into the structure and nature of the cytoskeleton as well as furthers the knowledge of the relationship between protein function and cytoskeletal linkage. For example, ultrastructural studies of the lymphocyte cytoskeleton revealed
that following detergent treatment, the surface topography of the lymphocyte was remarkably smooth and lacked microvilli compared to that of an intact γδ T cell, which consisted of numerous microlli (compare Figures 4.2A and B). Furthermore, detergent-treated cells were smaller in size. Previous measurements of bovine γδ T cells demonstrated a uniform size of approximately 4 μm (Leid et al., 1998), whereas detergent-treated γδ T cells were 3 μm in the studies described here. TEM provided additional information regarding the nature of the lymphocyte cytoskeleton. As previously reported, bovine γδ T cells had numerous microvilli, an intact plasmalemma and nuclear envelope, and various organelles including mitochondria and Golgi complex (Leid et al., 1998). By TEM, detergent-treated γδ T cells consisted of a nucleus, little or no cytoplasm, and a plasmalemma that had collapsed upon the nucleus. The detergent-treated γδ T cell lacked microvilli, while the plasmalemma and nuclear envelope remained partially intact. Thus, the detergent-treated lymphocyte consisted primarily of a nucleus surrounded by the plasmalemma.

In an attempt to better understand the functional consequences of L-selectin protein association with the cytoskeleton, its linkage was compared to the linkage of 28 other human and bovine surface antigens. From our flow cytometry and gel electrophoresis studies, we collated the nature of cytoskeletal association into three distinct patterns; 1) constitutive, 2) inductive, and 3) antibody crosslink-induced association. Human lymphocyte CD44 and bovine lymphocyte WC1 antigens, both
reported to act as signal transduction molecules, associate with the cytoskeleton prior to detergent treatment. This result is similar to previous reports that CD44 associates with lipid rafts and generates cellular activation signals utilizing the signaling machinery of the plasma membrane microdomains (Ilangumaran et al., 1999). CD44 binds hyaluronate and mediates the binding of lymphocytes to specialized high endothelial venules (Aruffo et al., 1990). Antibodies against CD44 induce homotypic cell aggregation as well as augment binding of human leukocytes to fibronectin (Cao et al., 1995 and Cao et al., 1996-7). Also, anti-CD44 mAbs or native CD44 ligands activate several signaling pathways that culminate in cell proliferation, cytokine secretion, chemokine gene expression and cytolytic effector functions (reviewed in Ilangumaran et al., 1999). Thus, it is clear that engagement of CD44 can lead directly to activation of lymphocytes (Jackson et al., 1992 and Aruffo et al., 1990).

WC1 is a lineage specific protein on bovine γδ T cells that can be expressed as 3 different isoforms, depending on specific gene rearrangements that take place (Davis et al., 1987, Clevers et al., 1990, and Winjgaard et al., 1992). The extracellular domain of the protein contains 11 homologous regions that have strong identity to the scavenger receptor cysteine-rich (SRCR) family (Winjgaard, et al., 1994). Scavenger receptors react with host serum proteins and may participate in host defense against bacterial infection (Chiang et al., 1995). Recently, WC1 has been suggested to control reversible γδ T cell growth arrest in an IL-2 dependent manner (Takamatsu et al., 1997, Kirkham, et al.,
1997 and Kirkham et al., 1998). Steroids can also affect the expression of WC1 on the surface of the γδ T cell. *In vivo* treatment with dexamethasone causes WC1 downregulation from the cell surface (Burton and Kehrli, 1996). Thus, it is clear that WC1 has the potential to act directly as a signal transduction molecule without co-stimulation, although studies are still under way to elucidate more about how this protein functions.

Other proteins found to be constitutively linked to the cytoskeleton include human CD4 and CD8, bovine CD4 (to a small extent by SDS-PAGE), CD5, CD8, CD18 (to a small extent by SDS-PAGE), CD45RO, and a yet undefined antigen on bovine monocytes recognized by mAb BN1-80 (Tables 4.1 and 4.2). Most of these proteins share the similar trait of signal transduction molecules, that upon engagement, cause activation of the lymphocyte. Although there is a difference between molecules such as CD44 and WC1, which have not yet been shown to require accessory molecule engagement for lymphocyte activation, and CD4, CD5, and CD8, it is apparent that all can transduce signals. Thus, our studies of the various proteins above suggest cell surface signal transduction proteins are constitutively linked to the actin cytoskeleton after syndissertation and transport, and linkage to the cytoskeleton provides a scaffolding for requisite protein function. In addition to these proteins, other proteins can transduce signals as well, but their linkage to the cytoskeleton may be regulated by ligand engagement.
The T cell receptor for antigen is important in recognition of foreign antigens and an intact cytoskeleton is vital for lymphocyte activation and function (Penninger and Crabtree, 1999). Upon activation, the TCR undergoes cytoskeletal rearrangement and associates closely with other important antigen recognition proteins such as CD2, CD3, and CD5, forming supramolecular activation clusters (SMACs) that aid in directing the cellular immune response (Monks et al., 1997, Monks et al., 1998, and Shaw and Dustin, 1997). Therefore, we investigated the cytoskeletal association of some of these proteins to further investigate the patterns of association as it related to their defined functions. For example, we have studied CD3, a co-stimulatory molecule that associates with the αβ and γδ TCR, and the TCR itself. These lymphocyte cell surface markers are not constitutively linked to the cytoskeleton but can be linked by mAb engagement alone. It is possible that some primary mAbs may cause crosslinking alone, however, this seems unlikely because three different anti-γδ TCR mAbs produced similar results. Recent work has shown a role for lipid rafts in B cell antigen receptor (BCR) signaling and antigen targeting and aggregation of lipid rafts has been shown to accompany signaling via the TCR (Cheng et al., 1999, and Janes et al., 1999). Both of these results were generated by crosslinking, however, and our data suggests that the bovine γδ TCR does not need to be crosslinked to associate with the cytoskeleton, as opposed to the human αβ TCR, or the BCR. This may be due to different reagents used in the studies that may bind more functionally important epitopes on the γδ TCR versus the αβ TCR. However, in many
cases, different mAbs directed against the same surface antigen were used and gave similar results suggesting that this was likely not the case. A more reasonable explanation is the fundamental difference of antigen recognition between an αβ T cell and a γδ T cell. Numerous studies have demonstrated that γδ T cells can recognize antigens without the aid of classical accessory molecules, such as MHC. Thus, engagement of the γδ TCR with mAbs may mimic ligand engagement \textit{in vitro} and \textit{in vivo}. Future studies will help define the uniqueness of primary antibody cytoskeletal induction of the γδ TCR as well as further characterize the features of γδ TCR association with lipid rafts, and γδ T cell function in general.

CD19 and CD45RA in humans, and CD2 and GD 3.5, a bovine γδ T cell specific antigen, also can be induced to link to the cytoskeleton (Tables 4.1 and 4.2). The distinction between the bovine γδ T cell specific antigens WCl and GD 3.5 may provide clues to the function of GD 3.5 antigen, and is important as their respective cytoskeletal association patterns further distinguish these molecules of similar molecular weight. Also, E-selectin ligand(s) were induced to associate with the cytoskeleton by engagement with recombinant E-selectin/Ig chimera (Tables 4.1 and 4.2). Some differences were seen between the cytoskeletal association of human and bovine CD2 and CD45RA. For example, CD2 could be induced to associate with the cytoskeleton by mAb engagement alone on bovine lymphocytes whereas it had to be crosslinked on human lymphocytes. Also, bovine CD45RA was not constitutively linked to the cytoskeleton, in contrast to
CD45RA in humans. These differences may be a result of the developmental stage of the lymphocytes from the animal tested. Young calves are immunologically immature, compared to adult human donors whose circulating lymphocytes are functionally mature. Therefore some of the proteins on human lymphocytes may have reached a developmentally mature stage which is characteristic of CD45RA constitutive cytoskeletal linkage. The CD2 result may also be explained in a similar manner.

A previous report demonstrated that L-selectin dynamically associates with the cytoskeleton upon mAb crosslinking, hyperthermic treatment, and Glycam-1 binding (Evans et al., 1999). Here, as with Evans and colleagues, we also show that L-selectin is linked to the cytoskeleton by mAb crosslinking. We have previously characterized a conformational change in L-selectin, caused by an anti-L-selectin mAb, that predisposes L-selectin to cytoskeletal association when a functionally distinct epitope is engaged by a separate anti-L-selectin mAb (Chapter 3). We believe that induction through a conformational change may more accurately represent a physiological process similar to native ligand binding. Indeed, as mentioned above, Glycam-1, a defined ligand for L-selectin, can induce L-selectin association directly (Evans et al., 1999). Therefore, ligand binding, which can be mimicked by mAb binding, may cause a conformational change in L-selectin, exposing a separate conserved epitope to a greater extent, and predisposing L-selectin to associate with the cytoskeleton. In turn, cytoskeletal association allows for tighter tethering and rolling interactions, slowing the lymphocyte rolling velocity, and allowing the L-selectin captured cell to more closely sample the microenvironment.
Hence, without the ability to associate with the cytoskeleton, strong L-selectin-mediated leukocyte capture can not take place. However, this possibility cannot be effectively studied at this time as both mAb and ligand binding block the function of L-selectin under flow. Nevertheless, L-selectin mutants that lack the 11 terminal amino acids of the cytoplasmic tail, do not associate with the cytoskeleton, and do not establish efficient rolling interactions \textit{in vivo} and \textit{in vitro}, suggesting a causal role between L-selectin cytoskeletal linkage and function (Kansas et al., 1993, Evans et al., 1999).

Similar to the genetic manipulation of the cytoplasmic tail of L-selectin, polyunsaturated fatty acids also inhibit the ability of L-selectin to associate with the cytoskeleton, while two other patterns of cytoskeletal association, constitutive and inductive, are not altered. Importantly, PUFA treatment also inhibited cytoskeletal association of tandem mAb treatment which may mimic direct ligand binding. This suggests that the machinery that mediates L-selectin cytoskeletal association is different than the other two types of proteins. Importantly though, PUFAs disrupt the cell membrane while keeping L-selectin intact and this system may provide a more physiologically relevant tool in understanding L-selectin cytoskeletal association \textit{in vivo}. Indeed, studies have shown that diets supplemented with PUFAs have anti-inflammatory effects which may be explained by a decrease in L-selectin, as well as E-selectin ligand-mediated selective recruitment of lymphocytes to sites of inflammation (Li and Steiner, 1991, and Calder, 1998). These issues are currently under investigation. The mechanism of PUFA action on L-selectin expression and cytoskeletal association remains unclear but
is similar to other published reports which demonstrate a reduction in adhesion molecule expression upon PUFA treatment as well as a reduction in protein association with the cytoskeleton (Sethi et al., 1996, Stulnig et al., 1998, and Hughes and Pinder, 2000). For L-selectin, PUFA treatment most likely blocks the interaction of α-actinin binding to actin by altering the spatial arrangement of these two proteins in the cell. This could explain the differences in the effects seen with varying degrees of unsaturation of fatty acids as eladic acid, a PUFA with a single degree of unsaturation, did not exhibit as dramatic an effect than other PUFAs with higher degrees of unsaturation (data not shown).

We have investigated a variety of surface proteins on human and bovine lymphocytes in an effort to provide more clues as to the function of L-selectin cytoskeletal association. Among other things, we have further described the nature of the lymphocyte cytoskeleton and compared its appearance and internal makeup to that of intact, non-detergent-treated cells. Also, these data suggest that adhesion molecules involved in rolling, i.e. L-selectin and E-selectin ligand(s), are induced to associate with the actin-based cytoskeleton upon mAb or ligand engagement and this may play a role in their ability to induce strong tethers. As the mechanisms underlying cytoskeletal association are further elucidated, more patterns of protein association with the actin-based cytoskeletal network, as well as the consequences of disrupting this linkage, will emerge, and provide additional information linking the cytoskeleton to overall cell function.


This dissertation describes the novel regulation of L-selectin by mAb, cytoskeletal association, and fatty acid membrane content. L-selectin is expressed on the tips of the microvilli on human leukocytes (Picker et al., 1991 and Bruehl et al., 1996). Here, we extend these observations to bovine lymphocytes (Leid et al., 1998). Transmission electron microscopy demonstrated that L-selectin is located at the tips of the microvilli of γδ T cells as visualized by anti-L-selectin mAb DREG 56 and colloidal-gold conjugated goat anti-mouse second stage staining. Scanning electron microscopy demonstrated that bovine γδ T cells contain greater than twofold the number of microvilli than αβ T cells and that bovine γδ T cells are smaller in size (4 μm versus 6 μm). It has previously been shown that bovine γδ T cells express 2-5 times the amount of L-selectin versus αβ T cells (Walcheck et al., 1994). We confirmed this finding here and suggest that because of the location of L-selectin at the tips of the microvilli, γδ T cells express more L-selectin simply because these cells contain more microvilli than αβ T cells. Differences in the size and surface topography of γδ and αβ T cells may be important in the interaction of these
cells with other host cells, such as endothelial cells, or targets they intend to kill, such as tumor cells or infectious agents.

A recent report demonstrates that L-selectin associates with the cytoskeleton by mAb crosslinking, hyperthermic treatment, and Glycam-1 binding (Evans et al., 1999). We have characterized a mAb-induced (LAM1-116) conformational change in L-selectin that predisposed the protein to cytoskeletal association. In the presence of this structural change in L-selectin, a second functional epitope on L-selectin, recognized by mAb EL-246, is upregulated, and when it is engaged by EL-246, cytoskeletal association occurs. The conformational change and ultimate cytoskeletal association of L-selectin was specific to these two antibodies as other anti-L-selectin mAbs, as well as other cell surface antigens, did not trigger cytoskeletal association. It is interesting to note that both mAbs recognize distinct functional epitopes on L-selectin and only these two epitopes were involved in non-mAb-crosslinked-induced L-selectin cytoskeletal association. The functional importance of the conformational change and eventual cytoskeletal association described is currently under investigation. Unfortunately, both mAb and native ligand, which all induce cytoskeletal association, also block the function of L-selectin. However, other work done in different systems have suggested a causal link between L-selectin cytoskeletal association and function.

L-selectin presumably associates with the cytoskeleton through binding of α-actinin to the cytoplasmic tail of L-selectin and actin. Previous work demonstrates that
the cytoplasmic tail is important in the function and regulation of L-selectin (Kansas et al., 1993 and Kahn et al., 1998) and transfectants lacking the 11 terminal amino acid residues of L-selectin do not roll in vitro and in vivo (Kansas et al., 1993). A separate study further concluded that L-selectin on these same transfectants does not associate with the cytoskeleton under any conditions (Evans et al., 1999). Therefore, it is implied that cytoskeletal association of L-selectin is important in mediating L-selectin rolling events.

The cytoskeleton is vital to lymphocyte activation and function (Penninger and Crabtree, 1999). To further investigate the lymphocyte cytoskeleton, as well as L-selectin cytoskeletal association and its relation to protein function, we compared the cytoskeletal association of L-selectin to that of 28 other cell surface proteins on human and bovine lymphocytes. Interestingly, scanning electron microscopy demonstrates that low dose detergent treatment leaves a highly uniform lymphocyte cytoskeleton with a remarkably smooth surface compared to an intact, non-detergent-treated γδ T cell. Detergent treatment of bovine lymphocytes strips the cell of its microvilli, or surface projections from the plasmalemma. Also, the lymphocyte cytoskeleton is smaller in size than an intact γδ T cell. A previous report has shown that bovine γδ T cells are approximately 4 μm in size. Here, we show that detergent treatment reduces the size of the bovine γδ T cell by ~25%, resulting in a detergent-treated lymphocyte of approximately 3 μm. Upon additional investigation by transmission electron
microscopy, detergent treatment of bovine lymphocytes demonstrates that the plasmalemma colapses around the nucleus, exhibiting little or no cytoplasm. In addition, detergent treatment leaves the cell without any obvious organelles or microvilli extending from the plasmalemma. As with a previous report, an intact, non-detergent lymphocyte retained these characteristics, including the presence of mitochondria and Golgi in the cytoplasm.

Three different types of protein cytoskeletal association are described herein, constitutive, inductive, and mAb crosslink-induced. Each characterization depends upon the timing of low dose detergent treatment to the cells compared to mAb treatment. Proteins constitutively associated with the cytoskeleton are visualized by mAb staining after detergent treatment. Some of these proteins included CD44 in the human and WC1 and CD5 in the cow. All of the proteins discovered to be constitutively linked to the cytoskeleton in these studies can act directly as signaling molecules, thus suggesting that constitutive cytoskeletal linkage is important in a cell's ability to transduce effective signals. Indeed, when the ability of some proteins to associate with the cytoskeleton is inhibited, efficient signaling does not take place (Stulnig et al., 1998). Other proteins are induced to associate with the cytoskeleton by primary mAb binding alone. These included molecules such as CD3, E-selectin ligand(s), and bovine γδ TCR. Under certain conditions, L-selectin cytoskeletal association mirrors that of E-selectin ligands, in that both are induced to associate with the cytoskeleton by direct ligand and/or
counterreceptor binding. L-selectin can also be induced to associate with the cytoskeleton by mAb crosslinking. Thus, for L-selectin, the ability to associate with the cytoskeleton depends upon a specific conformation of L-selectin. L-selectin can be induced to associate with the cytoskeleton by direct ligand binding or by dual anti-L-selectin mAb treatment that causes a conformational change in L-selectin, predisposing the protein to associate with the cytoskeleton when a distinct, conserved epitope recognized by mAb EL-246, is engaged. Taken together, these data suggest that cytoskeletal association provides a mechanism of regulation between various proteins and their receptors. Indeed, when these interactions are disrupted by incorporation of PUFAs into the cell membrane, the adhesive quality of L-selectin is dramatically reduced.

In summary, this work describes the novel alteration of L-selectin by mAb, cytoskeletal association, and fatty acid membrane content. A ligand mimicking mAb, LAM1-116, induces a conformational change in L-selectin, increasing the expression of a conserved epitope recognized by a distinct mAb, EL-246, and predisposes L-selectin to cytoskeletal association when the EL-246 epitope is engaged. Cytoskeletal association of L-selectin is important in its function as an adhesion molecule. By describing the regulation of an important epitope on L-selectin (EL-246), it may help design a better therapeutic agent during ischemia and reperfusion injury, as well as other shear dependent events. In the past, EL-246 has been shown to be an effective reagent against these injuries and now, with a better understanding of its interaction with L-selectin, may lead
to the next generation of reagents suited to fight both acute and chronic inflammatory events.

**Collaborative Work During My Graduate Program**


This paper describes the unusual expression of an E-selectin-like molecule on human hematopoietic cell lines by the staining of a variety of functionally important anti-E-selectin mAbs. It also demonstrates that the E-selectin-like protein can be immunoprecipitated from the cell membrane lysates of these human hematopoietic cell lines.


This paper describes the novel killing mechanism of an antibody that recognizes a cell surface protein on an equine protozoal myeloencephalitis parasite. Co-culture of the parasite with the mAb results in parasite death within 4 hours and total killing within 48 hours.
References Cited


