Molecular characterization of the primary adhesion mechanisms that direct [gamma/delta] T cells to epithelial-associated tissues
by Bruce Kenneth Walcheck

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Science
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Abstract:
γ/δ T cells, unlike α/β T cells, do not preferentially accumulate in secondary lymphoid tissues, such as the lymph nodes. Here, two questions are addressed: first, how do bovine γ/δ T cells exclude themselves from peripheral lymph nodes? The primary adhesion mechanism that directs α/β T cells to this lymphoid compartment involves the lymphocyte homing receptor L-selectin and the vascular addressin MECA-79 antigen. I show that this adhesion mechanism is highly conserved at the molecular level in the ruminant. In addition, bovine γ/δ T cells express functional L-selectin and at levels 2-5x greater than a/p T cells. γ/δ T cells, however, do not downregulate their L-selectin as efficiently as a/p T cells. Kinetic analysis revealed that at all time points after PMA activation (1-30 minutes), L-selectin expression remained significantly higher on γ/δ T cells and was downregulated at a slower rate compared with α/β T cells. A diminished capacity to downregulate L-selectin may disrupt the dynamics of extravasation by γ/δ T cells into lymph nodes. Second, what primary adhesion mechanisms direct bovine γ/δ T cells to epithelial locations? I show that 'γ/&delta T cells bind the skin vascular addressin E-selectin in vitro and in vivo. This adhesion event is dependent on divalent cations and can be abrogated by treatment of the γ/δ T cells with neuraminidase or proteases. The cell-surface adhesion proteins CD44, CD 18, and L-selectin on bovine γ/δ T cells are not involved in binding E-selectin. In addition, previously described carbohydrate ligands for E-selectin are not expressed by the γ/delta; T cells. An E-selectin affinity column purifies a single glycoprotein of 250 kDa from NP-40-solublized, γ/δ T cell membrane proteins, and, like γ/δ T cells, its binding is sensitive to neuraminidase digestion or the presence of EDTA. Based on theses observations, a mechanism is proposed for the primary adhesion events that selectively direct γ/δ T cells to epithelial locations.

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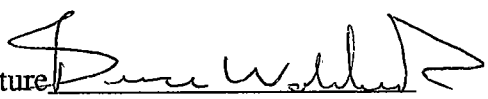
Signature 

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I dedicate this thesis to my parents Kenneth and Priscilla Walcheck, and my fiancee Cara Bird.

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ABSTRACT

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CHAPTER 1

INTRODUCTION

Historical Beginnings Of Lymphocyte Trafficking

Over thirty years ago, James Gowans' revolutionary studies of the recirculatory patterns of lymphocytes led to the modern day "understanding" of leukocyte trafficking patterns (1,2). Gowans' hypotheses were based on controversial observations from previous investigators, such as Davis and Carlson (1909) (3), and Sjovall (1936) (4), who theorized that lymphocytes exit the blood through the endothelial lining and enter the lymph. After World War II, studies pertaining to the life-span of lymphocytes, as well as the introduction of cannulation techniques and radioactive manipulation, provided Gowans with the means to more definitively examine lymphocyte recirculation. For instance, McGregor and Gowans (1963) (5) demonstrated that elimination of the recirculating pool of lymphocytes from the thoracic duct abrogated a primary immune response to sheep red blood cells or skin grafts, and reinfusion of lymphocytes restored the immune response. Gowans' initial studies performed in the rat were confirmed by Morris and Hall (1964) (6,7) using sheep. Due to the advantages of large animals, such as the accessibility of lymph vessels for cannulation and the larger numbers of leukocytes that can be harvested, exquisite studies have been performed (reviewed in ref. 8) revealing the very diverse nature

of lymphocyte trafficking. Indeed, lymphocyte trafficking patterns are influenced by specific tissues, the particular lymphocyte subset, and inflammatory events, as described below.

Tissue-selective lymphocyte trafficking

Following Gowans' original descriptions of lymphocyte recirculation, several lines of experimental evidence have demonstrated that blood to lymph trafficking is tissue-selective. Griscelli et al. (9-11) found that radiolabeled rat lymphocytes from the mesenteric lymph node exhibited a slight preference for gut-associated lymphoid tissues, whereas lymphocytes from peripheral lymph nodes (PLN) demonstrated a slight preference for lymph nodes following intravenous (iv) infusion into a syngeneic host. These studies were substantiated by Eugene Butcher et al. (12) using lymphocytes isolated from mouse lymphoid tissues in short-term homing experiments. Importantly, it is generally considered that lymphocytes harvested from lymphoid organs (by tissue disruption and cell suspension) recirculate in a random manner, similar to the cells in peripheral blood, whereas a greater proportion of the lymphocytes collected from lymphatics demonstrate nonrandom recirculation selective for the tissue of isolation (13,14). Only after analysis of cells isolated from lymphatic fluid of large animals were clear differences in the homing of certain lymphocyte subsets seen (15). Lymphocytes isolated from the efferent lymph of the sheep mesenteric lymph nodes preferentially migrate back to mucosal tissues after reinjection back into the animal. Likewise, cells isolated from the efferent lymph of PLNs preferentially migrate back to PLNs (16-18). These observations are not due to a dilution effect (19) or differential retention of labeled cells in different tissues (20).

The nonrandom lymphocyte migration continues beyond the one round of recirculation measured in most in vivo migration assays. Abernathy and Hay used an innovative method

of dual labeling cells to show that certain lymphocytes continuously recirculate in a tissue-selective fashion (21). Lymphocytes were harvested from sheep gut efferent lymphatics, labeled with FITC, and reinjected back into the animal. The intestinal lymph was collected again, lymphocytes were labeled with XRITC and then reinjected back into the animal. Two-color flow cytometry (FACS analysis) was used to analyze the cells that accumulated in the lymphatic fluid of the intestines and PLNs after the second injection. Cells labeled with either XRITC or FITC alone demonstrated a preferential accumulation in the intestinal lymph compared to preformal efferent lymph. However, double-labeled lymphocytes while showing similar kinetics of migration exhibited a greater degree of selectivity for intestinal tissues compared to the single-labeled populations.

Blast/memory (discussed in greater detail below) cells appear to represent the lymphocyte population that exhibits tissue-selective trafficking properties in lymphatic fluid and blood. This has been eloquently demonstrated by Mackay et al. (22). In this study, sheep lymphocytes, which were collected from the gut-draining lymphatics, labeled with FITC, and infused back into the syngeneic host, demonstrated a preferential reappearance in the gut versus lymph node lymphatics. The $FITC^+$ lymphocytes that recirculated back to the gut were phenotyped with antibodies against subset and differentiation markers and found to predominantly consist of cells expressing the blast/memory cell phenotype.

Lymphocyte subset-specific homing

Distinct subsets of lymphocytes exist; for instance, γ/δ T cells, α/β T cells (consisting of CD4 and CD8 lymphocytes) and B cells (consisting of B-1 and B-2 cells). Prior to antigen-dependent lymphocyte differentiation, naive lymphocytes demonstrate preferential migration pathways that are developmentally predetermined. This migratory characteristic has been shown in vivo for B cells and α/β T lymphocytes, and is independent of the organ

source of lymphocytes. B cells as a whole preferentially migrate to mucosal lymphoid tissues, whereas α/β T cells prefer PLN (though differences were subtle) (23-25). Within the α/β T cell population, in vivo migration assays have revealed a slight preference of $CD8^+$ T cells for PLN, as opposed to Peyer's patch for $CD4^+$ T cells (26). Another example of subset-specific homing is that demonstrated by the γ/δ T cell population (27). Unlike α/β T cells, γ/δ T cells do not preferentially recirculate through secondary lymphoid tissues, but exhibit a preference for epithelial cell-associated locations, such as the skin and mucosal sites (see below).

Furthermore, it appears that certain subsets of lymphocytes are more efficient than others in extravasation through the blood vascular wall (28,29). For example, independent of the site of collection (intestinal as well as lymph node afferent or efferent lymph) $CD4^+$ T cells' kinetics of recirculation is better than $CD8^+$ T cells (29-31). These results could not be explained by the selective retention of $CD8^+$ T cells in the parenchyma of the lymphoid tissue (32). $CD4^+$ T cells have also been shown in both sheep and rodent models to extravasate better than B cells (29,33). Interestingly, though only a small fraction of peripheral blood γ/δ T cells in sheep are capable of extravasating into lymph nodes, the cells that do extravasate seem to transit at the same rate as $CD4^+$ T cells (28).

Inflammation-specific lymphocyte migration

In addition to their recirculation through secondary lymphoid organs, lymphocytes also migrate into sites of inflammation. For example, Chin and Hay (19) examined the migratory patterns of lymphocytes through sites of chronic inflammation. These authors induced the inflammatory event by subcutaneous injection of Freund's complete adjuvant into the hind limbs of sheep, then collected lymphocytes by cannulation of the afferent lymphatics draining the site of inflammation, radiolabeled these cells, and injected them iv

back into the animal. They found that the radiolabeled lymphocytes nonrandomly migrated to the inflammatory lesion. Similar findings were reported by Issekutz in the same animal model (34). These studies indicate that lymphocyte migration into sites of inflammation exhibit levels of specificity comparable to that seen in lymphocyte recirculation. Not surprisingly, lymphocyte subsets that preferentially home to inflamed tissues exhibit memory cell phenotypes (35,36).

As summarized in Table 1, the physiology of lymphocyte recirculation has been well addressed by several research groups in a variety of animal models. This, however, leaves

Table 1. Lymphocyte tissue-selective recirculatory pathways.

Tissue sites of recirculation	References
1. PLNs	(15,16,18,41)
2. Gut-associated lymphoid tissue	(16-18,41)
3. Lung-associated lymphoid tissue	(41,48)
4. Spleen (?)	(41)
5. Skin	(21,36,41,92)
6. Gut lamina propria	(41,170)
7. Sites of inflammation	
a. skin	(19,34,36,41,92)
b. synovium	(41,49)

in question the molecular mechanisms that allow lymphocytes to accumulate in the various tissues. Gowans and Knight (37) noted the association of lymphocytes with morphologically distinct vessels known as postcapillary high endothelial cell venules and

postulated potential "homing" mechanisms for lymphocytes.

Lymphocyte/Endothelial Cell Adhesive Interactions In Recirculation

Previous studies using myeloid cells have revealed that leukocyte extravasation is a complex process involving at least three steps that represent an association of signalling events (by cell-cell interactions and various cytokines) and adhesion events (by primary and secondary adhesion proteins) (38,39). Together these mechanisms function as a combinational process resulting in very specific tissue and site extravasation by leukocytes. The steps are 1) primary adhesion, a reversible interaction represented by rolling of the leukocytes along the vascular wall; 2) secondary adhesion, a stronger adhesive interaction that allows the leukocyte to arrest on the vascular wall; and 3) transmigration. Recently, through manipulation of signalling cascades and adhesive mechanisms, lymphocyte migration into certain tissues has been shown to involve multiple steps as well (40). In my thesis, I will predominantly concentrate on step 1, the primary adhesive mechanisms, used in lymphocyte extravasation and, to a lesser degree, steps 2 and 3.

The extravasation of lymphocytes from blood into the extravascular tissue involves the recognition of specialized postcapillary venules (PCV), referred to as high endothelial venules (HEV) in human and mouse secondary lymphoid tissues (37,41,42). The lymphocyte/PCV interaction has been demonstrated by in vivo homing studies and the Stamper and Woodruff ex vivo frozen section binding assay (43) in which lymphocytes discriminate, with remarkable specificity, PCV in different tissue sites. For example, mouse intraepithelial lymphocytes or lamina propria lymphoblasts adhere to HEV in mucosal lymphoid tissue, but not in PLNs (44-46). Overall, unique lymphocyte/PCV interactions have been described in PLNs (46,47), Peyer's patch (44-46), peribronchial

lymph nodes (48), and at sites of inflammation (41,49,50).

The ability of lymphocytes to discriminate between the PCV of secondary lymphoid and nonlymphoid tissues is directed by the interaction between lymphocyte receptors (homing receptors) and endothelial cell ligands (vascular addressins) (14). A primary adhesion event occurs when the lymphocyte's tissue-selective homing receptor recognizes an endothelial cell addressin. Homing receptors and vascular addressins used for distinct tissue-selective trafficking pathways have been best described for the PLNs, gut, and skin. L-selectin, α4/β7, and CLA represent defined lymphocyte homing receptors for PLN, gut, and skin, respectively, whereas MECA-79 Ag, MadCAM-1, and E-selectin represent defined vascular addressins in PLN, gut, and skin, respectively (summarized in Table 2).

Table 2. Specific homing receptor/vascular addressin combinations at various tissue sites.

Tissue site	Adhesion protein expressed by....	
	Lymphocytes	Endothelial cells of PCV
PLNs	L-selectin	MECA-79 Ag
Gut-associated lymphoid tissue	α4β7	MadCAM-1
Skin	CLA	E-selectin

L-selectin/MECA-79 Ag

L-selectin, the PLN homing receptor, is the most thoroughly characterized tissue-selective adhesion protein (51). The MEL-14 mAb (51) in the mouse and Leu-8 (52), TQ-1 (52), LAM-1 (53), and the DREG series of mAbs (54) in the human all recognize L-

selectin, and block lymphocyte binding to PLN venules in vitro as well as in vivo. L-selectin is a member of a family of adhesion proteins known as Selectins (other members include E-selectin and P-selectin, see below) derived from evolutionary-related genes on human and mouse chromosome one (55). L-selectin is a 70-110 kDa (depending on the leukocyte subset) integral glycoprotein comprised of a series of extracellular domains homologous to mammalian type-C lectins, EGF, and complement binding proteins (which vary in number depending on the particular family member). L-selectin also has a conventional transmembrane region and a short cytoplasmic tail (52,53,56-58).

L-selectin has unique functional and regulatory characteristics. Previous studies have shown that the functional capacity of L-selectin is served by its Ca^{+2}-dependent lectin domain, which recognizes specific anionic polysaccharides. SLex (a sialylated lactosamine) (59), mannose-6-phosphate (60), a mannose-6-phosphate-rich yeast phosphomannan called PPME (61), and the fucose sulfate-rich polysaccharide known as fucoidin (60) represent anionic sugars known to interact with L-selectin and block function.

Lymphocyte expression of L-selectin appears to be regulated in two distinct manners. First, lymphocyte L-selectin is regulated in a short-term manner, which results in rapid cleavage of the molecule from the cell surface. The activating agent phorbol 12-myristrate 13-actetate (PMA) induces rapid (within 15 minutes) downregulation of lymphocyte L-selectin expression (54,62-64), which is blocked by inhibitors of protein kinase C (63), with a return to near baseline levels at 24 hours (65). This process can be enhanced by a calcium ionophore, resulting in a more complete loss of L-selectin (64); however, subsequent reexpression does not occur (65). Treatment with the ionophore alone results in a slow reduction in L-selectin levels over several days (64,65).

Rapid L-selectin downregulation appears to occur solely by shedding of the molecule from the cell surface (64), which is mediated by a putative membrane-bound,

chymotrypsin-like protease that cleaves L-selectin just extracellular of the transmembrane region (66,67), though the critical protease remains to be defined. Other signal transduction pathways, such as those involving G-proteins, have not been shown to be involved in rapid lymphocyte L-selectin downregulation. However, studies by Palecanda et al. have shown that L-selectin/ligand interactions alone mediate rapid L-selectin shedding independent of overt activation, in vitro and in vivo (68). Regardless of the actual mechanism that leads to rapid L-selectin shedding, the process itself is believed to be important in lymphocyte detachment following primary adhesion allowing the cell to proceed with transmigration through the blood vessel wall via secondary adhesive processes.

A second mechanism of L-selectin regulation on lymphocytes occurs during blastogenesis (the transition from naive to blast/memory cells) within lymphoid compartments. Previous studies have shown that lymphocytes activated in vitro with mitogens, anti-CD3, and alloantigens in vivo downregulate surface L-selectin expression (69). Following lymphocyte activation in vitro with Con A or anti-CD3, the activated cells transiently upregulate surface expression of L-selectin in the first 24 hours (69); though, by day 2, L-selectin expression begins to decrease, reaching a complete loss by day 6 (69,70). However, upon removal of the stimulating agent, blast cells enter a resting state and L-selectin expression returns (71). L-selectin mRNA levels parallel surface expression following anti-CD3 stimulation (69). In contrast, PMA activation of a T cell line (Jurkat), which caused rapid L-selectin shedding, significantly increased L-selectin mRNA levels; however, levels slowly decreased if PMA and a calcium ionophore (or the calcium ionophore alone) were present, (65). Thus, unlike short-term activation, lymphocyte activation through the TCR/CD3 complex results in changes in L-selectin expression which is regulated by changes in mRNA levels.

A recent study by Picker et al. (72) has demonstrated the relevance of long-term L-selectin regulation during lymphocyte blastogenesis at sites of PLNs versus mucosal lymphoid tissue. Conventional naive lymphocytes are homogeneous in L-selectin expression (73). Once a naive lymphocyte encounters its specific antigen in a lymphoid organ, the cell undergoes a stepwise transition (blastogenesis) through distinct phenotypic intermediates culminating in a blast/memory phenotype ($CD45RA^{low}/CD45RO^{high}$, among other markers). Similar to the in vitro findings, L-selectin expression during this transition is uniformily upregulated on blast cells; however, in mucosal lymphoid tissues L-selectin is then preferentially downregulated, whereas in PLNs the high level of expression is retained (72). Thus, blast/memory lymphocytes exhibit bimodal L-selectin expression (42). This process assures tissue-selective homing to the lymphoid tissue in which the lymphocyte first encounters antigen. For instance, human and mouse intestinal lymphoblasts selectively migrate to mucosal tissues and are L-selectin negative (43,45).

PLN HEV express the L-selectin ligand (vascular addressin) which has been defined by the mAb MECA-79 in the human and mouse (74,75) [MECA 79 also stains bovine (Jutila personal observation) and sheep PLN PCV (22)]. The MECA-79 antigen, detected by Western blotting, represents a lymph node-specific (under normal conditions) but heterogeneous glycoprotein containing of O- and N-linked carbohydrates. The molecular weight of the MECA-79 Ag is approximately 90 kDa, but additional minor bands of 50, 70, 150, and 180 kDa have been noted, suggesting a possible post-translational modification that decorates multiple glycoproteins.

Overall, three separate PLN PCV ligands for L-selectin have been described; GlyCAM-1 (Sgp50), GlyCAM-2 (CD34 or Sgp90), and $PNAd^{gp90}$. GlyCAM-1 and 2 are mucin-like glycoproteins possessing of sulfur, fucose, and sialic acid (59,76), and both are recognized by MECA-79 (77). GlyCAM-2 represents a glycoform of the endothelial cell-

surface protein CD34 (78). PNAd^{gp90}, another MECA-79 Ag, has an approximate molecular weight of 90 kDa, similar to GlyCAM-2; however, the relationship between these two ligands remains undetermined. The PLN vascular addressin in my thesis will be referred to as the "MECA-79 Ag". MECA-79 inhibits normal lymphocyte binding to PLN HEV ex vivo and lymphocyte trafficking to PLN in vivo (74). MECA-79 reactivity with vessels in tissues of chronic inflammation has also been noted, though the significance of this on lymphocyte recruitment is unknown. For instance, chronic inflammatory lesions in the skin show MECA-79 reactivity; however, the kinetics of its expression is believed to occur after the expression of another vascular adhesion protein, E-selectin, which is involved in the recruitment of various leukocyte subsets (79).

α4β7/MadCAM

The integrin family consists of heterodimeric molecules that are composed of an α subunit noncovalently associated with a β subunit. Several distinct α subunits can associate with only one β subunit. In addition, certain α subunits can associate with different β subunits. Based on the shared β subunits, subfamilies can be distinguished within the integrin family. Only molecules belonging to the β1, β2, and β7 subfamilies are expressed on lymphocytes (80).

The α4β7 (LPAM-1) and α4β1 (VLA-4) integrins have been proposed to play a role as homing receptors for mucosal sites (81). Antibodies against the mouse lymphocyte α4-chain were shown to selectively inhibit lymphocyte binding to Peyer's patch HEV in the ex vivo assay (81,82) and, in the rat, lymphocyte trafficking to the Peyer's patch but not PLNs in vivo (83). Furthermore, trafficking to these sites in the mouse has been shown to be blocked at a similar level by mAbs directed towards the α4β7 dimer or β7 itself. In contrast, an antibody to the β1-chain had no influence on lymphocyte trafficking to mucosal

sites, suggesting a selective role for the α4β7-dimer in lymphocyte trafficking to mucosal sites (84).

In addition to the Peyer's patches, migration into the lamina propria itself is also affected by anti-α4 and -β7 antibodies, predicting an overlap in trafficking mechanisms that guide cells into the gut lymphoid tissue and into the intestinal mucosa (84,85). Importantly, naive lymphocytes migrate only at a low rate into the lamina propria in comparison to Peyer's patch trafficking; however, lymphoblasts display a preferential migration into the intestinal mucosa, possibly due to acquired tissue-selective properties derived during lymphoid education (41).

The mucosal vascular addressin MadCAM-1 is a 58-66 kDa glycoprotein and a member of the immunoglobulin and mucin-like families of adhesion receptors (86). MadCAM-1 is selectively expressed on the PCV in mucosal lymphoid organs (Peyer's patch and MLN) and lamina propria (85,87), and it is recognized by the mAb MECA-367 which is capable of blocking lymphocyte recognition of intestinal PCV in vitro and in vivo (85). MadCAM-1 is also a ligand for the α4β7-integrin, but not α4β1 (88). α4β1, and possibly α4β7, recognize VCAM-1 (a cytokine-inducible ligand expressed on endothelial cells at sites of inflammation) and fibronectin as well; however, these ligands are not believed to play a role in lymphocyte trafficking to noninflamed tissues (84). Thus, the α4 integrins appear to play a dual role in lymphocyte trafficking into either mucosal tissues or into sites of inflammation

In addition to the α4 integrins, previous data suggest that L-selectin may also play a small role in lymphocyte trafficking to mucosal tissues (84,89,90). Significant inhibition of Peyer's patch localization has been observed when MEL-14 is co-injected with lymphocytes during the in vivo assays, and complete inhibition has been observed when anti-α4 and anti-L-selectin mAbs are used in combination (84). In support of these

observations, reactivity of the MECA-79 mAb has been detected at low levels on Peyer's patch HEV (though induction of MECA-79 reactivity due to inflammation cannot be ruled out) (22,89-92). In addition, the MECA-79 mAb recognizes a subset of MadCAM-1 isolated from MLN, and the same MadCAM-1 can support L-selectin-dependent lymphocyte rolling in in vitro flow assays (93).

CLA/E-selectin

The Selectin family members E- and P-selectin are inducible adhesion proteins expressed by activated endothelial cells (though the kinetics of expression are considerably different) and are involved in primary adhesion with a variety of leukocyte subsets (94,95).

E-selectin has been implicated in a dual role for the acquisition of leukocytes into inflamed tissue. E-selectin expression was originally described on acutely inflamed endothelium in virtually all extralymphoid tissues for the recruitment of PMNs (96-98). Subsequently, it has been shown to be preferentially induced at sites of chronic inflammation in the skin (psoriasis, contact dermatitis, nonspecific dermatitis, etc.) that are associated with infiltrates of mononuclear cells (79,99). Furthermore, Norton et al. have suggested that particular tissues may constitutively express low levels of E-selectin on some venules (67,68). Recent studies have shown that human CD4 memory T cells, but not naive cells, avidly bind E-selectin (79,99). The capacity to bind E-selectin following the acquisition of the memory phenotype is associated with the expression of the mAb HECA-452 epitope (CLA antigen) on T cells (36,100). HECA-452 recognizes the "face" of the carbohydrate moiety on lymphocytes that is bound by E-selectin (101). It has been suggested that the interaction between memory T cells and E-selectin may be important in the preferential accumulation of these lymphocytes in certain inflammatory sites (36,79,99). Picker et al. have extended this hypothesis, based on their observation that E-selectin

appears to be preferentially expressed on venules in the skin, and proposed that E-selectin serves as a vascular addressin for lymphocyte recirculation through dermal tissues (36,79).

A previously described component of leukocyte receptors for E-selectin consists of specific carbohydrate moieties (similar to L-selectin ligands, see above). These sugars are composed of sialylated and fucosylated lactosamines from the Lewis family, such as sLex, sLea, and other undefined members recognized by the HECA-452 mAb (102).

The carbohydrate ligands appear only to be a partial representation of the entire receptor for selectins. It is likely that the biological receptors for the selectins would also be composed of lipid or protein. Importantly, both P- and L-selectin have been shown to bind specific cell-surface glycoproteins with high affinity (91,103). The complete significance of this protein component has not been determined. Protein may provide for a "scaffolding" in which carbohydrate structures are presented to the selectins (91). However, it is possible that certain regions of the protein backbone may participate in a protein-protein interaction with the selectins, in addition to the carbohydrate-lectin interaction.

Studies addressing the nature of the biological receptor on leukocytes for adhesion to E-selectin vary considerably in their findings. SLex and the HECA-452 Ag carbohydrate structures are found on many different glycoproteins and glycolipids on the leukocyte surface, suggesting that a large number of molecules could potentially serve as receptors (104); however, this does not appear to be the case. Activation of neutrophils actually increases sLex expression, but binding to E-selectin is greatly diminished (104-106). Thus, there appears to be a restriction in the types of molecules on the leukocyte surface that can serve as E-selectin receptors.

Putative glycoprotein receptors for E-selectin have been described on neutrophils. L-selectin is one surface protein that may preferentially present sLex on neutrophils to E-selectin. Anti-L-selectin mAbs block neutrophil adhesion to E-selectin cDNA transfectants

to the same extent as anti-E-selectin mAbs, suggesting that E- and L-selectin can serve as receptor ligand pairs (105). Picker et al. extended these observations and found that L-selectin is decorated by sLex, and its preferential localization at the tips of the microvilli and ruffles of the neutrophil cell-surface membrane may account for its predominant role in presenting carbohydrates to E-selectin (104). In a recent study, Vestweber et al. (107) have described two additional glycoproteins isolated by an E-selectin affinity technique. From detergent lysates of mouse myeloid cells and bone marrow cells, a 160 kDa molecule was identified; however, from the bone marrow cells, an additional 250 kDa molecule was noted which may have been derived from the lymphocyte progenitor cells. Other cell-surface glycoproteins have been proposed as possible E-selectin ligands as well, such as lysosomal membrane glycoprotein-1 (87-120 kDa) (108), leukosialin (CD43, 120 kDa) (108), CD66 [90 kDa (PI-linked) or 160 kDa (transmembrane)] (109), and the integrin CD11/CD18 (CD11=150-177 kDa, CD18=95 kDa) (110); however, their biological relevance has yet to be determined. Importantly, an analogous role for L-selectin or any other single glycoprotein in mediating lymphocyte adhesion to E-selectin has not been shown.

Secondary adhesive mechanisms

As alluded to above, the migration of lymphocytes from blood into extravascular tissues involves multiple steps. Following primary adhesion, the mechanisms that lymphocytes use to strongly attach to the endothelium and transmigrate involve several secondary adhesion proteins (38,39). These include various integrins and immunoglobulin family members on lymphocytes and endothelial cells, respectively, such as: LFA-1/ICAM-1 and ICAM-2, and VLA-4/VCAM-1. Other interactions that appear to be involved are between lymphocyte CD44 and integrins binding to certain extracellular matrix components (111).

γ/δ T Cells

Lymphocyte recirculation pathways and the molecular mechanisms that direct these cells have been well addressed by many research groups. However, the current understanding of lymphocyte trafficking is based upon conventional lymphocytes, such as α/β T cells, but little is known about γ/δ T cells. A reason for this is that γ/δ T cells were initially identified at the DNA/mRNA level and only recently have cell-surface lineage-specific markers been identified. Summarized below is the current understanding of this functionally enigmatic T cell lineage.

γ/δ T cells (reviewed in 112) have been found in all vertebrates examined so far, including humans (113), chickens (114), mice (115), rats (116), ruminants (27), and pigs (117). γ/δ T cells resemble α/β T cells in terms of development, structure, and functional capabilities. Like α/β T cells, most γ/δ T cells are derived from precursors that enter the thymus where they differentiate and, during fetal development, begin to colonize peripheral tissues. The γ/δ TCR is heterodimeric, usually disulfide-linked, and coupled in a functional manner to the CD3 complex, which appears to have the same subunit composition as the CD3 complex on α/β T cells (118). The genomic organization of gamma and delta genes is similar to that of alpha and beta genes, and, via the same mechanisms that generate diversity in α/β T cells, γ/δ T cells can potentially express a wide array of γ/δ TCR proteins that are capable of specific ligand recognition. Many of the genes that can encode the variable region of delta chains are either identical or very similar in their nucleotide sequences to alpha chain variable region genes (119). The alpha and delta variable region genes are also physically linked--evidence that they are probably descendents of the same ancestral genes (120). The genes encoding gamma and beta proteins are not linked, but are structurally alike (120). Due to genetic similarities, the ligand binding sites of γ/δ antigen receptors

probably resemble those of α/β antigen receptors and should permit similar receptor ligand interactions. Indeed, γ/δ T cell clones have been isolated that recognize MHC class I- and class II-related molecules (120,121). Furthermore, certain γ/δ T cell clones have been shown to respond specifically to small peptide regions that are also recognized by MHC restricted α/β T cells. (120)

Despite the similarities between γ/δ and α/β T cells, significant differences do exist. For instance, in the mouse, fetal thymus rearrangement and surface expression of γ/δ genes precede those of α/β genes (112,122). Interestingly, nonproductive gamma gene rearrangements are common in α/β T cells (123), which may suggest that α/β T cells are derived from T cell progenitors that failed to express γ/δ TCR (124). The thymic passage by γ/δ T cells is much faster than α/β T cells (125), indicating they follow a less complicated maturational pathway, though there is evidence of positive and negative selection (112). During thymic ontogeny, γ/δ T cells traverse the mouse fetal thymus as single, developmentally programmed waves (expressing distinct γ/δ TCR receptors with each wave) to eventually seed their target tissue (126).

γ/δ T cells can express many of the cell surface proteins that α/β T cells do, such as CD2, CD3, CD4, CD5, CD7, CD8, CD11b, CD16, CD25, CD28, or CD45; however, the frequency of the cells expressing a particular protein and the level of expression vary widely, not only between α/β and γ/δ T cells, but also between subsets of γ/δ T cells and, importantly, between γ/δ T cells of different species (112). γ/δ T cells isolated from human blood and murine skin share with natural killer (NK) cells the expression of Fc-gamma-receptors which mediate antibody-dependent cellular toxicity (112,127). The FcR on γ/δ T cells may broaden their scope of antigen recognition via bound IgG. In addition to the γ/δ TCR, a family of proteins known as Workshop Cluster 1 (WC1), also referred to as T19, are the only lineage-specific surface proteins on γ/δ T cells described thus far (128-131).

Anti-WC1 mAbs stain γ/δ T cells from ruminants (cow, sheep, goat), but not from mice or humans. Recently the cDNA from a member of the WC1 family was cloned and found to encode a transmembrane protein of 1436 amino acids (131). The molecule contains a large extracellular domain consisting of 11 cysteine-rich, scavenger receptor repeats that are typical of the family of proteins which includes CD5, CD6, and probably additional WC1 proteins (131).

Histocompatability protein restriction

A few murine and human γ/δ T cell clones have been shown to be specific for class I and class II proteins containing presented peptides (112). However, this appears to be the exception: Overall, γ/δ T cells do not demonstrate a bias towards classical class I and class II MHC. The majority of γ/δ T cells recognize peptides and perhaps other small molecules such as carbohydrates in association with nonpolymorphic antigen presenting proteins, such as class I molecules encoded in the TL and Q region of the class I locus or CD1, which is not linked to the MHC (112). Certain TL region gene products are expressed by epithelial cells at specific sites in close association with γ/δ T cells (112,132). Thus, various murine γ/δ T cell subsets may possibly recognize antigens that are presented by different tissue-specific TL region-encoded proteins. Most γ/δ T cells lack the expression of CD4 and CD8, which may be related to their tendency to recognize TL region-encoded class I molecules, since these molecules lack binding sites for the TCR coreceptors (133). Several human γ/δ T cell clones recognize a cell-surface protein referred to as T cell target antigen 1 (TCT.1 or CD48), a member of the immunoglobulin superfamily that is encoded by a gene located near the CD1 gene cluster (134).

Though a small fraction of γ/δ T cells express the CD4 marker, these cells are not believed to normally function as helper cells for B cells; however, B cell suppression is yet

to be resolved. No antibody response to T cell-dependent antigens was obtained in α/β T cell-depleted mice and rats (112,135), nor in α/β TCR-knockout mice (112). On a similar level, the γ/δ T cell population does not seem to contain many cells that recognize allogeneic MHC proteins (112). α/β T cell-depleted rats failed to reject skin grafts from MHC mismatched donors (136). Thus, the context of antigen recognition and, perhaps, effector function of γ/δ T cells seems to be markedly different from α/β T cells.

Host defense

The functional capabilities of γ/δ T cells resemble α/β T cells, NK cells, and lymphokine activated killer cells in that they can lyse target cells and express the same granule mediators of cytotoxity, such as perforin and serine esterase 1 and 2 (112,137). γ/δ T cells also accumulate within regions associated with inflammation; for instance, in the draining lymph nodes of mice infected in the footpad with mycobacteria (138), in the lungs of mice infected intranasally with influenza virus (139), in the peritoneal cavity of mice infected with *Listeria monocytogenes* (140-142), in hepatic granulomas of Schistosome infected mice (143), and in the skin lesions of patients with the tuberculous form of leprosy or with cutaneous leishmaniasis (144,145). Elevated numbers of γ/δ T cells have been noted in the spleens of mice infected with *Trypanosoma cruzi* and *Plasmodium chabaudi* (146), in the blood of patients during the acute and recovering phases of malaria infections (112,147), and in the acute phase of Epstein-Barr virus infection (112). Elevated numbers of circulating γ/δ T cells have been reported in the blood of HIV-infected patients, as well (112,148).

In contrast to the beneficial functions γ/δ T cells may serve to the host, these cells appear to associate with and may contribute to pathological immune responses in several different diseases; for example, rheumatoid arthritis (149), collagen-induced arthritis (150),

atopic dermatitis (151), pulmonary sarcoidosis (152), Lupus erythematosis (153), and scleroderma (154).

The role γ/δ T cells serve in host defense and the kinetics of their responsiveness are far from understood, though increasingly sophisticated studies are being performed to address this. For instance, genetically deleted or antibody depleted γ/δ and/or α/β T cell animals are being used, which are challenged in some manner and examined for alterations in immune responsiveness compared with normal animals. Using such techniques, three recent reports have examined the differential roles of γ/δ and α/β T cells during primary and secondary infection by the intracellular bacterium *Listeria monocytogenes* in mice (140-142).

Hiromatsu et al. (140) provided evidence that γ/δ T cells precede α/β T cells in appearance during primary infection with *Listeria.* The responding γ/δ T cells proliferated and secreted certain cytokines in in vitro assays to antigens, such as purified protein derivative (PPD) and heat-shock protein-65 (HSP-65), but not to heat-killed *Listeria.* Importantly, following systemic γ/δ T cell depletion by a specific mAb, an increased number of bacteria was evident in the spleen at the early stage of *Listeria* infection. α/β T cell-depleted mice demonstrated similar resistance as control mice, thus demonstrating a functional importance of γ/δ T cells early in infection. *Listeria,* however, were eventually eliminated in γ/δ T cell-depleted mice, similar to control mice, while an appreciable number of bacteria persisted in the spleens of α/β T cell-depleted mice (at day 12). These results were expanded upon by Skeen et al. (141) who showed γ/δ T cells, collected from the site of primary *Listeria* infection and restimulated in vitro, exhibited a greater expansion potential than the α/β T cells. However, the memory response in *Listeria*-immunized mice was virtually unaffected by depletion of γ/δ T cells. Recently, Mombaerts et al. (142) performed similar experiments in mice that were deleted of α/β and/or γ/δ T cells by germ-line mutations. In these mice, it was confirmed that primary infection was eliminated in

either α/β or γ/δ T cell deleted-mice, though kinetically *Listeria* persisted for a longer period of time. In agreement with Skeen et al. (141), resistance to secondary infection was mediated mainly by α/β T cells, but appeared to involve γ/δ T cells to a small degree. Along these lines, a delayed-type hypersensitivity response to heat-killed *Listeria* was dependent on α/β T cells and not γ/δ T cells. In addition to the different immune responsiveness in α/β or γ/δ T cell-depleted mice, typical granulamatous lesion formation was altered as well. Histological findings suggest that α/β T cells were necessary for the development of granulomas, though γ/δ T cells appeared to regulate α/β T cells, and in their absence large abscessed lesions were noted.

A significant fraction of γ/δ T cells appear to recognize antigens that are shared between microbes and mammals, which include viral proteins (155), carbohydrates (156), and HSPs (155). PPD of mycobacterium represents a well-described γ/δ T cell antigen, and is composed of a large fraction of HSP-65 (157). HSPs, which function within stressed cells in part to chaperon and help fold proteins, are among the most abundant and most conserved polypeptides of the biosphere (158).

Studies using small synthetic peptides corresponding to various portions of mycobacterial HSP-65 identified a portion of the protein, amino acids 180-196, which is evolutionarily highly conserved. Mouse γ/δ T cell clones recognized not only the mycobacterial sequence but also equivalent sequences, of homologous proteins in other species, including autologous protein (159). A major γ/δ T cell subset (Vg9/Vd2 in human and Vg1 in mice) can respond to the mycobacterial HSP peptide, which resembles superantigen recognition by certain subsets of α/β T cells (120). In addition to the mycobacterial HSP, γ/δ T cell stimulatory components were found in a low molecular weight fraction (2-10 kD) of mycobacterial extracts; these components were resistant to proteolytic enzymes and are capable of binding lectins (112). However, further characterization of this stimulant remains to be performed (including whether it is of

mycobacterial or host-cell origin).

As established by the above studies, γ/δ T cells appear to serve primarily as a first line of defense preceding α/β T cells during microbial host response until clonally expanded numbers can respond. γ/δ T cells may play a role in surveillance against tumors as well, as suggested by the isolation of γ/δ T cell lines from populations of tumor infiltrating lymphocytes (112,160).

Tissue-selective trafficking

In all animals, a fundamental difference between γ/δ and α/β T cells is their primary site of accumulation (112): For α/β T cells, after leaving the thymus, this represents the secondary lymphoid organs such as PLN and Peyer's patch. γ/δ T cells, which typically represent only 1-5% of the cells in lymphoid organs (27,129), accumulate in nonlymphoid sites--particularly epithelial-associated tissues, such as skin and mucosal tissues (27,161,162). The γ/δ T cell's inherent nonlymphoid migration appears to be consistent between newborn and mature animals (27). Furthermore, studies in mice and humans suggest that unlike the α/β T cell's heterogeneous TCR composition in different tissues, γ/δ T cells segregate into tissue-specific subsets with homogeneous TCR phenotypes. For instance, in mice the Vγ5 and Vγ1 TCR phenotypes are predominant in the skin, Vγ6 in the vagina, uterus, and tongue, and Vγ7 and Vγ1 in the intestinal tract (112). In humans Vδ1 is predominantly found in the thymus and intestinal tract, whereas Vδ2 is found in the peripheral blood (112,163). Thus, γ/δ T cells seem to be fixed during ontogeny to particular tissues, rather than being shaped by antigenic challenge and clonal selection, as is characteristic of α/β T cells.

γ/δ T cell numbers in various tissues range greatly between different animals. For instances, in humans and rodents these cells typically represent <5% of the T cells in

peripheral blood (164), whereas in ruminants (cattle and sheep) they can represent up to 50% of the mononuclear cells (45). In ruminants and humans, γ/δ T cells are found in the dermis of the skin, but in mice the cells appear to be mostly restricted to the epidermis (128;165). Moreover, in mice (165) and chickens (166) γ/δ T cells represent the dominant intestinal intraepithelial lymphocyte population, but in cattle these cells are mostly found in the lamina propria (27). Importantly, in all species γ/δ T cell numbers in the various tissues are affected by age and antigen stimulation.

Summary

Lymphocytes recirculate via the blood and lymph to distribute themselves throughout the tissues of the body in an attempt to encounter specific antigens. Two general recirculation pathways are used by lymphocytes; 1) lymphoid, in which lymphocytes recirculate from the blood through various secondary lymphoid organs; and 2) nonlymphoid, in which lymphocytes recirculate from the blood through various peripheral tissues. Naive α/β T cells recirculate exclusively by the lymphoid route through all lymphoid compartments. Only after antigen stimulation and blastogenesis do α/β T cells develop the capacity to selectively recirculate through the lymphoid compartment of initial antigen recognition, such as PLN, and certain nonlymphoid tissues. *Unlike α/β T cells, γ/δ T cells demonstrate a developmentally predetermined ability to recirculate through nonlymphoid tissues, such as epithelial-associated sites like the skin and mucosal tissues, but not lymphoid organs. The γ/δ T cell's unique migration pattern correlates with their described functional role in the immune system, which is as a first line of defense.*

The epithelial tissue-selective recirculation pattern of γ/δ T cells introduces two important questions which have not previously been addressed. First, how do these cells exclude themselves from secondary lymphoid tissues? An examination of the expression of the known adhesion molecules involved in lymphocyte recirculation may reveal an inherent lack of lymphoid homing receptors and/or the necessary secondary adhesion molecules. Additionally, adhesion protein regulatory mechanisms may be altered, affecting blood vessel transmigration. The well characterized PLN recirculatory event by α/β T cells provides an ideal system for comparison to determine which adhesion-associated mechanism(s) is (are) different on γ/δ T cells; for instance, γ/δ T cells may lack L-selectin expression, similar to blast/memory α/β lymphocytes which selectively traffic through non-PLN sites. Second, what molecular mechanisms control γ/δ T cell entry into epithelial locations? Though many studies have demonstrated the phenomena of peripheral seeding and recirculation by γ/δ T cells, the actual molecular events controlling their selective trafficking to epithelial tissues have not been characterized. The following chapters represent an accumulation of experimental data that begin to address these questions. Because the different mammalian species are considerably variable in γ/δ T cell numbers, an appropriate animal model was chosen to optimize the ability to study these cells.

Animal model

The ideal source of "homing-competent" γ/δ T cells is the peripheral blood, since these cells are in the process of trafficking and their repertoire of adhesion-associated receptors have not been altered by emigration, which is not the case for lymphocytes harvested from solid tissues (83,167). However, the study of γ/δ T-cell trafficking in humans and mice is problematic, since these cells represent a minor population of the T cells in peripheral blood (typically <5%). In contrast, ruminants (cattle and sheep) contain a substantial fraction of

these cells in their circulatory system. In the immature animal, the γ/δ T-cell population may represent up to 50% of the peripheral blood mononuclear cells, typically declining to 10-25% as the animal matures (45,168). In addition, these cells represent a significant population of the lymphocytes that recirculate through epithelial sites in the immature and mature animal. Thus, much of what is known about the biology of γ/δ T cell trafficking has come from studies of sheep and cattle (27). Importantly, cattle possess three prominent T-cell populations: 1) $CD2^+$ $CD4^+$ $CD8^-$, 2) $CD2^+$ $CD4^-$ $CD8^+$, and 3) $CD2^-$ $CD4^-$ $CD8^-$ (null cells) (169). The CD2 lineage marker denotes α/β T cells, where the null cells predominantly represent γ/δ T cells. As discussed above, ruminant γ/δ T cells can also be identified by the anti-WC1 antibodies, which are more effective than anti-TCR antibodies due to their intense cell-surface staining by FACS and immunoperoxidase analysis (128,131). We, for the most part, used Holstein calves as an animal model to investigate the adhesion-related mechanisms that direct γ/δ T cells to epithelial locations.

In conclusion, understanding the molecular mechanisms involved in γ/δ T cell trafficking is essential for further examination of their function. Controlling their ability to localize within tissue could provide information of their role at that site, for instance at epithelial barriers, or in certain immune responses. Blocking specific tissue accumulation of γ/δ T cells, as opposed to systemic depletion by anti-TCR antibodies or genetic knockout techniques, provides a means of examining the role of γ/δ T cells at very localized sites without complete elimination of this immune cell component. Furthermore, the manipulation of γ/δ T cell trafficking could prove important in controlling pathological events such as rheumatoid arthritis, allergic diseases, and autoimmune disorders, such as systemic lupus erythematosis and scleroderma. On the other hand, by increasing the accumulation of γ/δ T cells, certain immune responses and therapies may be enhanced, such as vaccinations against particular infectious microorganisms (e.g., mycobacteria).

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CHAPTER 2

CHARACTERIZATION OF THE BOVINE PERIPHERAL LYMPH NODE HOMING RECEPTOR (L-SELECTIN)

Introduction

The continuous recirculation of lymphocytes via the vascular system and the lymphatics allows the full repertoire of antigenic specificities of the immune system to be represented throughout the lymphoid tissues of the body. The first step in the recirculation process is adhesion of the lymphocyte in the peripheral blood to unique endothelial cells found in lymphoid tissues and in some sites of chronic inflammation. Of particular importance is the striking tissue-selective feature of lymphocyte homing. Distinct tissue-selective molecular mechanisms regulate lymphocyte extravasation into mucosal versus peripheral lymphoid sites (1-3). The best characterized tissue-selective adhesion protein is the receptor involved in lymphocyte homing into peripheral lymph nodes (PLNs) (3). The MEL-14 mAb in the mouse (3) and a number of mAbs in the human, which include Leu-8 (4), TQ-1 (4), LAM-1 (5) and the DREG series of mAbs (6), recognize the PLN homing receptor. These mAbs block lymphocyte adhesion to PLN venules, but have little effect on adhesion to venules in mucosal tissues. cDNAs encoding the PLN homing receptor of mice and humans have been cloned by a number of different laboratories (4,5,7-9), and predict a conventional

membrane glycoprotein made-up of domains homologous to regions of mammalian type-c lectins, epidermal growth factor, and complement binding proteins. The lectin activity of the PLN homing receptor is thought to be the regulating determinant in the interaction of this molecule with the vascular endothelium (8,10,11).

There are distinct advantages in working with larger animals for studies of lymphocyte trafficking. For example, tissue-selective populations can be readily isolated by harvesting the draining lymphatics of a given lymphoid tissue or simply harvesting the lymphoid tissue itself and isolating cells directly (12-19). The large size of the animal allows the collection of significant numbers of lymphocytes for structure/function analysis of specific adhesion proteins. We (20), and others (21), have shown that ruminant lymphocytes adhere readily to mouse HEV in the conventional Stamper and Woodruff *ex vivo* HEV binding assay. This is a significant advancement, since it shows that the large animal need only serve as the source of lymphocyte and not the substrate (endothelial cell) for tissue-selective adhesion studies. Finally, ruminant models for Leukocyte Adhesion Deficiency (LAD) and γ/δ T-cell studies have been recently described (22,23). Therefore, it is of considerable interest to begin to characterize ruminant lymphocyte adhesion proteins at the molecular level.

As far as we know, molecules homologous to human and mouse L-selectin have not been characterized in the cow. Here we show that 4 anti-human L-selectin antibodies crossreact with bovine leukocytes. We demonstrate that bovine L-selectin is functionally, biochemically, and molecularly similar to the human and mouse homologues. Differences were noted, however, which include 1) a much more tissue-restrictive expression of L-selectin in bovine tissues versus what has been described in the mouse and human, 2) the existence of an additional N-linked glycosylation site in the bovine molecule versus what has been predicted in the human and mouse, and 3) the shared expression of an antigenic

epitope on bovine leukocyte L-selectin and endothelial cells.

Materials and Methods

Animals

Cattle, which were of mixed breed and whose ages ranged from <1month to >1year, were purchased from local producers and housed at the Montana State University large animal facilities in the Veterinary Molecular Biology Laboratory. Peripheral blood was collected into heparinized tubes by venipuncture of the jugular and the leukocytes isolated by ficoll-hypaque (Histopaque 1077, Sigma, St Louis) separation. Tissue samples were collected from untreated animals upon necropsy. Synovial tissue samples were obtained from caprine arthritic encephalitis virus-positive arthritic goats.

Reagents

The mAb used in this report have been described in detail elsewhere. Leu-8, DREG-200, DREG-152 and DREG-56 are all mouse mAb that recognize human L-selectin (4,6). Hermes-3 (24-26) is a mouse mAb that recognizes CD44 (HCAM) on human leukocytes and crossreacts with bovine leukocytes. R15.1 is a mouse mAb that recognizes CD18 on human leukocytes (gift of Wayne Smith, Baylor University) and that also crossreacts in the cow (22). SH 43 and SH 77 are mouse mAb that we have recently raised and were used as negative controls in many of the experiments described below. Phorbol 12-myristrate 13-actetate (PMA) and chymotrypsin were purchased from Sigma (St Louis, MO).

Immunoperoxidase staining

Acetone-fixed 6 um frozen sections were incubated with antibodies in PBS (50 ug/ml) for 30 minutes at room temperature in a humidified chamber, and then washed in PBS. Using a TAGO histochemical kit (Histoprobe, TAGO, Burlingame, CA), a 3 stage immunoperoxidase stain using an avidin-biotin system was performed according to manufacturer's instructions. Sections were lightly counterstained with hematoxylin.

Immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis

Immunofluorescence staining of bovine leukocytes was carried out in 4ml tubes. Briefly, $1x10^6$ cells were initially incubated in 5% rabbit serum for 10 minutes on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50-100 ug/ml for 20 minutes on ice. After washing, bound antibodies were revealed by incubation with phycoerythrin (PE) or FITC conjugated F(ab)'2 goat anti-mouse Ig (Tago, Burlingame, CA) at a 1:80 dilution in 5% FBS in DMEM. FACS analysis was performed on a FACScan (Becton and Dickinson, Mountain View, CA) as described (6,27,28). For two color analysis, PE-conjugated Leu-8 (Becton Dickinson) and a FITC-conjugated DREG mAb were used. Negative control mouse mAbs were used to evaluate the level of background staining. Data were collected from 10,000-50,000 cells and are presented as the percentage of cells staining above background, mode fluorescence, or as histograms.

In vitro PMA and chymotrypsin treatment of bovine mononuclear cells

Isolated peripheral blood mononuclear cells were treated with PMA (10 ng/ml) for 15 minutes at 37°C in HBSS. After the incubation time the cells were fixed in 0.5% paraformaldehyde, washed, and then stained for FACS analysis. The effects of chymotrypsin were tested as previously described (29). Briefly, isolated mononuclear cells

were treated with different doses of the protease for varying periods of time at 37°C in HBSS. After each time point the cells were fixed in paraformaldehyde, washed, and stained for FACS analysis.

High endothelial cell venule (HEV) Assay

The *in vitro* assay of lymphocyte binding to HEVs in frozen sections has been extensively described (1,6,30,31). No modifications were done in order to measure bovine lymphocyte-HEV interactions. Like human lymphocytes (6), bovine lymphocytes bind specifically to mouse lymph node HEV. Cell binding was quantitated by first identifying HEVs in each field by their characteristic autofluorescence, and then counting cells bound to HEVs, as described (6). Each data point reflect triplicate sections; each section with 100-250 HEVs enumerated, and were from two separate experiments. Data were calculated as mean number of cells bound per individually scored HEV.

RNA isolation plus first strand cDNA generation

Peripheral blood leukocytes were collected from 200 ml of bovine or sheep peripheral blood. The blood was diluted 1:1 with Ca^{+2}, Mg^{+2} free HBSS and underlayed with Histopaque 1077 (Sigma). The preparation was spun at 2300 rpm for 30 minutes at room temperature. The buffy coat on top of the histopaque was collected and washed with HBSS. Cells were counted (4.5×10^8), pelleted, and then resuspended in 4 ml guanidine thiocyanate homogenization solution (4 M guanidine thiocyanate, 17mM sodium N-lauroylsarcosine, 24 mM sodium citrate) containing 8% 2-mercaptoethanol (v:v). Total cellular RNA was isolated from the homogenate via acid-phenol-chloroform according to Chomczynski et al. (32), 10 ug of which was used as template in synthesizing first strand cDNA using Superscript reverse transcriptase (20 ul reactions) according to Gibco BRL

(Grand Islands, New York), based on Kotewicz et al. (33). The cDNA reaction (10ul) was used directly in PCR cloning, described below.

PCR cloning of the bovine sheep L-selectin lectin domain

Oligonucleotide extension primer sequences were designed based upon the human L-selectin cDNA sequence. Primer sequences were selected from 5' and 3' (extra-domain) regions flanking the lectin domain (unique to the L-selectin molecule), that showed high similarity to the mouse L-selectin cDNA (Figure 1).

HR1 primer 5'GGAATTCTGGACAATGCTCTGTTGTGATTTC3'

HR2 primer 5'GGAATTCGCAAGAAGCTGTGTAACAGAGGGCTT3'

Figure 1. PCR oligonucleotide extension primer sequences were designed based upon the human L-selectin cDNA sequence. Primer sequences were selected from 5' and 3' extra-domain flanking regions to the lectin domain (unique to L-selectin and not the other Selectin family members, E-selectin and P-selectin), which showed high identity to the mouse L-selectin receptor cDNA.

Each primer contains 5' EcoRI restriction enzyme sites. PCR was done according to the established Taq polymerase protocol (Perkin Elmer, Norwalk, CT) based on (34-36). The following thermal profile was used: [(94° C 1 minute, 50° C 1 minute, 72° C 2 minutes)x35] cycles. The PCR product was run on a 1% agarose gel along with the PCR product in which human L-selectin cDNA (6) was used as template (+ control). The gel was stained with ethidium bromide and examined on a UV transilluminator. A second PCR

was done in which 5ul of the first reaction product was used as template to generate ample product for subcloning. The thermal profile was [(94° C 1 minute, 65° C 1 minute, 72° C 2 minutes)x25].

Sequencing of the PCR cloned bovine and sheep L-selectin lectin domain

The PCR product described above was EcoRI (New England Biolabs. Beverly, MA) and the fragment gel purified on a 1% agarose gel. The gel section containing the DNA fragment was frozen at -20° C for one hour and then liquefied at room temperature on parafilm. The DNA was separated from the agarose by a 1.5 ml Millipore (0.45 um) microfuge tube (Millipore Corp. Bedford, MA.) centrifuged at 10,000 g for 10 minutes. at room temperature, phenol (TE pH 8.0 saturated)/chloroform purified, and NaCl/ethanol precipitated.. The gel-purified product was ligated into the pBS SK+/- cloning vector (Stratagene, La Jolla, CA.) with T4 ligase (New England Biolabs). DH5 alpha competent cells were transformed with the plasmid-insert and colonies were selected by blue/white selection (Bluo-Gal (Gibco BRL) as substrate) according to the Gibco BRL protocol. Acid/phenol minipreps (modification of Maniatis et al. (37) were performed on several isolated colonies and the plasmid DNA digested with EcoR-1 to confirm the presence of the 426 bp PCR product. The plasmid DNA from three separate clones that contained the insert were sequenced, and double checked by opposing primers to reveal the sequence of the complementary strand, using a T7 Sequencing Kit (Pharmacia, Piscataway, NJ), based on Sanger et al. (38).

Results

Characterization of bovine leukocyte L-selectin

We initially showed that the anti-human L-selectin antibodies, DREG-56 (6), DREG-200 (6), DREG-152 (6) and Leu-8 (4) crossreact with bovine, but not goat or sheep, peripheral blood leukocytes. The antigen recognized by the anti-L-selectin mAb had a molecular weight of approximately 85 kD by non-reducing SDS-PAGE Western blot analysis (20 and data not shown). We next compared the level of expression of L-selectin with CD18 (common β_2 chain of the leukocyte integrins) on different subpopulations of bovine leukocytes from animals of different age (Table 3). Bovine neutrophils and monocytes stained brightly by anti-L-selectin and anti-CD18 antibodies (Table 3). Bovine lymphocytes expressed equivalent levels of L-selectin compared to neutrophils and monocytes, but their expression of CD18 was much lower (Table 3). Interestingly, staining

Table 3. Expression of the DREG-56 antigen and CD18 on bovine leukocytes.

Leukocyte[a]	Antigen	Percent positive[b]	Mode flourescence[c]
PMN	DREG	98±2.4[e]	233±63
	CD18	98±1.0	338±69
Monocyte	DREG	94±5.3	289±44
	CD18	99±0.4	496±111
Lymphocyte			
(<1month)[d]	DREG	88±4.1	214±21
	CD18	93±5.4	95±28
(>1year)	DREG	42±19.7	n.d.

a. Leukocytes were isolated from the peripheral blood and stained with DREG-56 and anti-CD18 mAbs. The bound primary antibodies were revealed by FITC-second stage. Background fluorescence was determined by staining with an irrelevant negative control.
b. Percent positive cells is the percentage of leukocytes that exhibited staining above the staining by the negative control antibody.
c. Mode fluorescence is the level of fluorescence of the positive cell populations after staining with the antibodies to the indicated antigens.
d. The expression of the DREG antigen was compared between lymphocytes isolated from animals <1 month and >1 year old.
e. Mean$\pm$sd (n= 7, for lymphocytes >1 year, n=6)

of bovine peripheral blood lymphocytes varied greatly with the age of the animal. In young calves (<1 month) 88.4$\pm$4.1% (n=7) of the lymphocytes were positive, whereas, in older animals (>1 year) the percentage of positive lymphocytes ranged from 17%-67% (42.2$\pm$19.7%, n=6). No differences were noted in the staining of myeloid cells from animals of varying age (data not shown).

A unique and distinguishing characteristic of L-selectin is that it is rapidly (within 15 minutes) released from the surface of the cell upon activation with PMA or physiological activating factors, such as chemoattractants (6,27,28,39,40). Here we tested the effects of PMA on the expression of L-selectin compared to CD18 and CD44 on bovine mononuclear cells (lymphocytes and monocytes). Within 15 minutes PMA treatment caused >70% reduction in L-selectin expression on bovine lymphocytes and monocytes (Figure 2). In contrast, CD18 expression went up in expression on both cell types and CD44 increased slightly on monocytes (Figure 2).

The release of L-selectin after activation appears to be due to a unique proteolytic process, since among other reasons, very low doses of chymotrypsin cause a specific release of the homing receptor molecule (29). Bovine lymphocytes treated with doses of 0.1 units/ml of chymotrypsin or higher for 15 minutes at 37°C exhibited a >90% loss of L-selectin expression (data not shown). We next tested the kinetics of this effect. Within 5

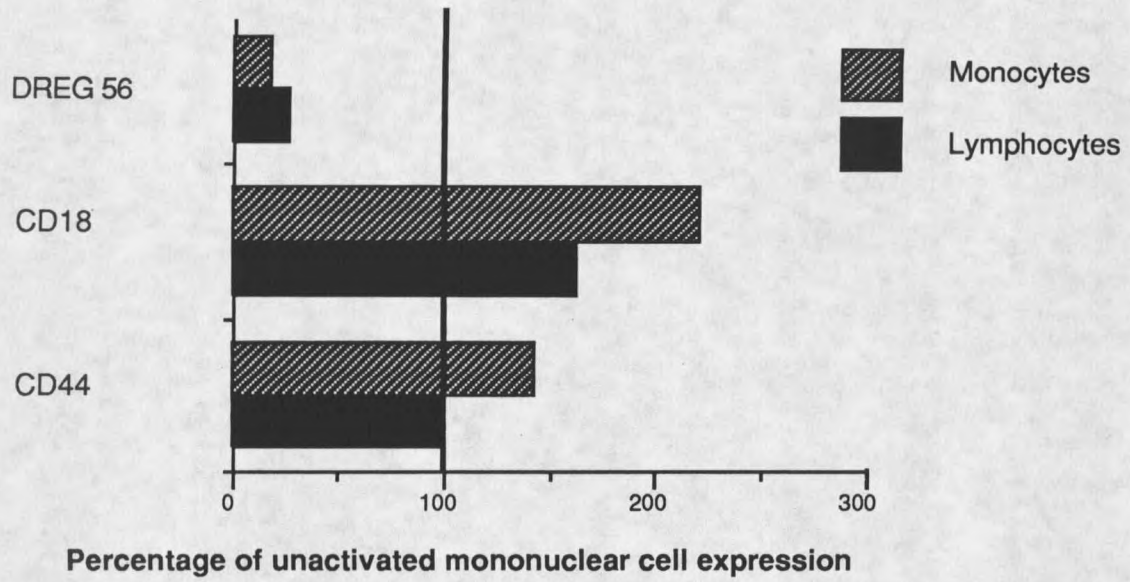

Figure 2. PMA activation of bovine mononuclear cells increased CD18, but decreased L-selectin expression. Bovine peripheral blood lymphocytes and monocytes were isolated, as described in Materials and Methods, and treated with 10 ng/ml PMA for 15 minutes at 37°C. The expression of the DREG-56 antigen, CD18, and CD44 was compared on activated versus unactivated monocytes and lymphocytes. The activated and control cells were stained with antibodies to the indicated antigens followed by the appropriate second stage, and flow cytometry performed. The data is presented as the percentage of control unactivated cell expression of the different surface antigens, where control (solid line) reflects 100%. The increase in CD18 expression after activation was not due to an increase in non-specific background fluorescence, since staining with an irrelevant control antibody did not increase (data not shown).

minutes after treatment with the protease, the percentage of L-selectin positive lymphocytes dropped from 58% to 44% (Figure 3). By 10 minutes the percent positive cells dropped to approximately 3%. Importantly, the few L-selectin positive cells that remained at 10 minutes expressed far less antigen than the cells at time zero (mode fluorescence 210 versus 460). Within 15 minutes L-selectin was apparently completely removed from the surface of virtually all of the cells (Figure 3). The loss of anti-L-selectin (DREG-56) staining was not

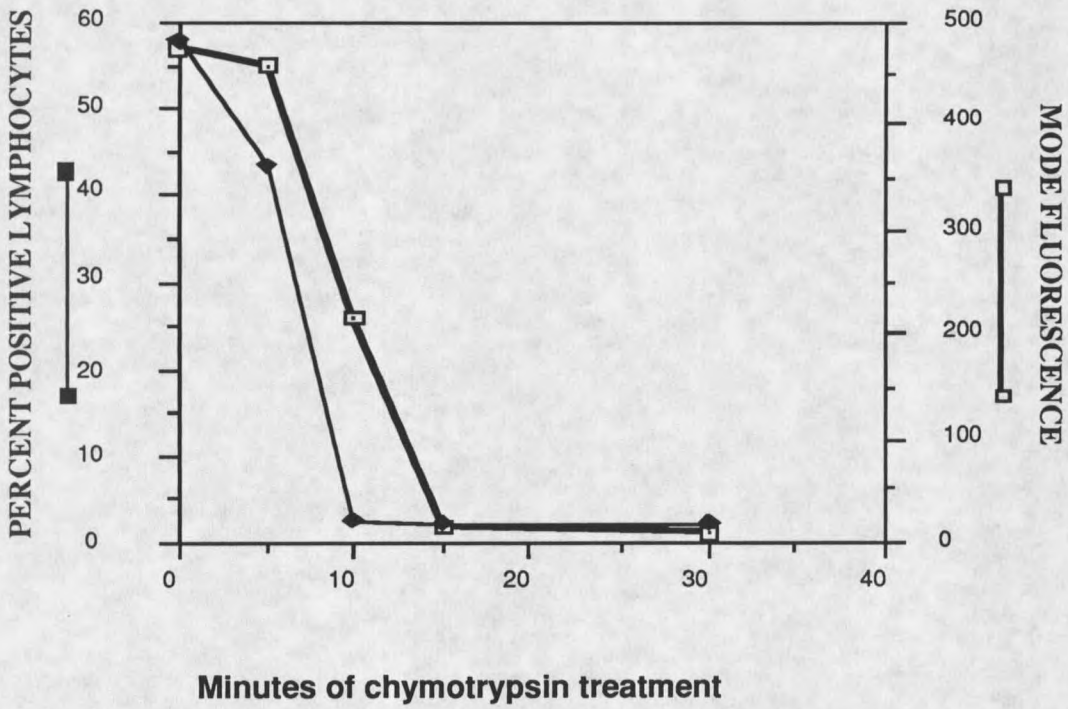

Figure 3. Low-dose chymotrypsin treatment of bovine lymphocytes resulted in a rapid loss of expression of bovine L-selectin. Bovine peripheral blood mononuclear cells were isolated, as described in Materials and Methods, and treated with 1 unit/ml chymotrypsin for different periods of time at 37°C. After the indicated time points, cells were fixed in paraformaldehyde and stained with DREG-56 followed by FITC-anti-mouse Ig second stage. Background fluorescence was determined by staining of an irrelevant control antibody. Both the percent positive cells (cells staining above background) and level of fluorescence (mode fluorescence) at each time point is presented. No changes in background staining were observed (data not shown). The experiment was repeated 3 times and the results are from a representative experiment.

epitope specific, since chymotrypsin also caused a decrease in DREG-200 and Leu-8 staining. Chymotrypsin had no effect on the expression of CD44 (data not shown).

We tested whether anti-L-selectin would block bovine lymphocyte adhesion to PLN HEV. We used mouse PLNs as a source of lymphoid tissues, because 1) bovine lymphocytes actively bind mouse HEV, 2) the molecular mechanisms controlling

lymphocyte-HEV interactions are conserved between species (41), 3) venules in murine lymphoid tissues that support lymphocyte adhesion are more readily identified, since they have a distinctive plump morphology not seen in bovine tissues, and 4) pathological inflammatory processes that occur in lymphoid tissues and which can alter interpretation of adhesion data are more readily controlled in mice than in cattle. We found that like human lymphocyte adhesion to mouse PLN HEV (6), bovine lymphocyte binding was blocked by the DREG-56 antibody by > 80% (Figure 4). A second anti-bovine lymphocyte antibody had no effect which showed that the blocking by DREG-56 was specific.

We cloned a region of both bovine and sheep L-selectin cDNA to further establish their level of similarity to the human and mouse homologue. We concentrated on just the lectin domain since Rosen and his colleagues have shown that this region of the molecule is critical for the interaction of L-selectin with the surface of the vascular endothelium (8,10,11). Total RNA was isolated from either bovine or sheep peripheral blood lymphocytes, cDNA prepared, and PCR performed utilizing extension primers based on the human L-selectin cDNA sequence. The primer sequences were unique to L-selectin (see Materials and Methods) to assure that the PCR product would indeed be the L-selectin lectin domain. A single band was observed on an ethidium bromide stained 1% agarose gel equivalent in size to the human L-selectin cDNA PCR product (positive control), (data not shown). After subcloning of the bovine or sheep PCR product, sequence analysis was performed on three independent clones from each animal due to the potential of PCR artifact. The nucleotide sequences of the three separate clones from each animal were identical. Comparison of the bovine lectin domain nucleotide sequence revealed a nucleotide identity of 84.2% and 80.4% with the human and mouse homologues, respectively (Figure 5). In addition, a comparable degree of identity between the predicted amino acid sequences

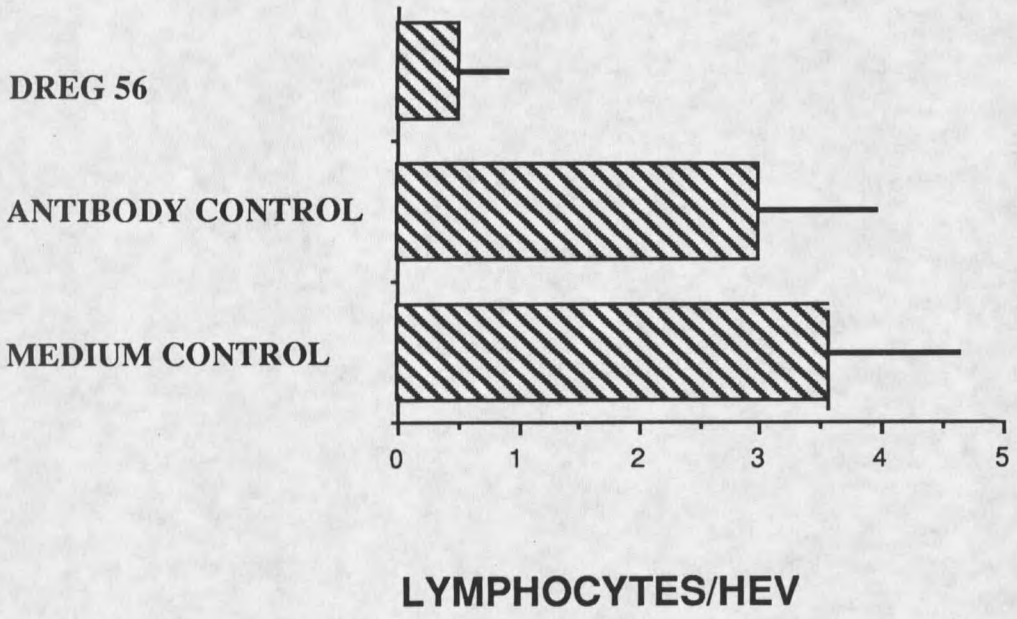

Figure 4. Anti-L-selectin antibody specifically blocked bovine lymphocyte adhesion to PLN HEV. Bovine peripheral blood lymphocytes were isolated, as described in the materials and methods, and used in the Stampard and Woodruff *ex vivo* HEV binding assay. Mouse PLNs were used as a source of HEV. DREG-56, but not an isotype control antibody, specifically blocked bovine lymphocyte adhesion to PLN HEV. The results reflect mean±sd.

[(based on the cDNA nucleotide sequence) of the bovine versus the human and mouse lectin domains] was seen (81.6% and 76.3%, respectively, Figure 6). Interestingly, bovine L-selectin was greater than 96% identical at the nucleotide and amino acid level to the sheep PCR product (Figures 5 and 6). Bosworth and co-workers have used our bovine lectin PCR product to clone the entire bovine L-selectin cDNA and have found a similar level of identity throughout the entire length of the molecule (Bosworth, B. and J. Harp, personal communication).


lectin domain
BOVINE 5' T TTGCTCATCGTGGCA CCGATTGC TGGACT 30
OVINE * ****G********** ******** ******
MOUSE C *GATA**C*A***A* *TC*C**T ******
HUMAN C *G**A****A***A* **T*C*** ******
TACCATTATTCTAAA AGACCCATGCCCTGG GAAAAGGCTAGAGCG 75
********** ********** ***G**********
**********G** *AG******AA***** *****T******AA*
**********G** *A*******AA***** C***G*******AGA
TTCTGCAGGGAAAAT TACACAGATTTAGTT GCCATACAAAACAAA 120
*********AG***** ********** **********G
*******A*C***** **********C **********G
******C*A**C*** ********** **********G
GGAGAGATCGAATAC CTGAATAAGACACTT CCCTTCAGCCGTACT 165
********** ********** ********T******
A****A**T**G*GT T*AG*G**T***T*G ***AAA****C*TA*
*CG**A**T**G**T ***G*G*****T**G ********T***T**
TACTACTGGATTGGA ATCCGGAAAGTAGAA GGGGTGTGGACTTGG 210
********** ********** **********
**********A*** ***A*****A*T*GG AAAA*******A***
**********A*** ********GA***G* **AA*A*****G***
GTGGGAACCAACAAA TCTCTCACTGAAGAA GCAAAGAACTGGGGT 255
********** ********** **********
********** A********A***** ***G**********
********** ********** ***G**********A
GCAGGGGAGCCCAAC AACAGGAAGAGTAAG GAGGACTGTGTGGAA 300
*A********** ********** **********
T**C******* ****A****TCC*** **********G
*AT**T********* ****A*****AC*** ********C*****G
ATCTATATCAAGAGG AACAAAGACTCGGGG AAATGGAATGATGAT 345
********** *T********** ********C******
********** GAACG******T*** ****CC**C*****C
**********A ********TG*A**C ********C*****C
GCCTGCCACAAAGCA AAGAAA XXXXXX 3' 366
********** ******
*****T******CG* ***GC*
**********CT* ***GC*

Figure 5. Comparison of bovine and sheep L-selectin lectin domain nucleotide sequence to the human and mouse L-selectin homologues. Relative conservation of nucleotides revealed a sequence identity of 84.2% between bovine and human and 80.4% between bovine and mouse. The last six nucleotides within the bovine lectin domain (XXXXXX) were included in the PCR extension primers and were not listed for the derived sequence.

The one notable difference in the bovine and sheep lectin domain was the predicted existence of a third N-linked glycosylation site in amino acid position 39 (Figure 6). The other two sites (Asn positions 22 and 66) were conserved between cow, sheep, mouse, and human.

Expression of L-selectin in bovine tissues

Using flow cytometry, the expression of L-selectin was compared on lymphocytes isolated from bovine PLNs (pre-scapular), mesenteric lymph nodes (MLN), and ileal Peyer's patch. These experiments were repeated three times and a representative result is shown in figure 7. Greater than 80% of the cells in PLNs and approximately 40-60% of the cells in MLNs were L-selectin positive. In contrast, <20% of small lymphocytes in the Peyer's patch express L-selectin (Figure 7). Immunohistological analysis showed that in PLNs all germinal center cells were negative for L-selectin, but most B-cells and T-cells of the follicles and paracortex, respectively, were positive. Much like what was found in the flow cytometric analysis described above, little if any DREG-56 staining could be detected on cells in the Peyer's patch and lamina propria (data not shown).

Immunohistological analysis revealed a novel tissue staining pattern of two of the anti-L-selectin mAbs. DREG-152 and DREG-200 reacted with cells closely associated with the vascular wall in a variety of diverse tissues. Both mAb faintly stained smooth muscle of large arteries in virtually all tissues tested, which included spleen, thymus, and lymph nodes. The unique staining of these two mAb was not restricted to cow, since similar

NH2-TERMINUS

BOVINE	**trp thr tyr his tyr ser lys arg pro met pro trp glu lys ala arg**	16
OVINE	*** *** *** *** *** *** *** *** *** *** *** *** *** glu *** ***	
HUMAN	*** *** *** *** *** *** glu lys *** *** asn *** gln arg *** ***	
MOUSE	*** *** *** *** *** *** glu lys *** *** asn *** *** asn *** ***	
	ala phe cys arg glu asn tyr thr asp leu val ala ile gln asn lys	32
	*** *** *** *** arg *** *** *** *** *** *** *** *** *** *** ***	
	arg *** *** *** asp *** *** *** *** *** *** *** *** *** *** ***	
	lys *** *** lys gln *** *** *** *** *** *** *** *** *** *** ***	
	gly glu ile glu tyr leu asn lys thr gln pro phe ser arg thr tyr	48
	*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***	
	ala *** *** *** *** *** glu *** *** leu *** *** *** *** ser ***	
	arg *** *** *** *** *** glu asn *** leu *** lys *** pro tyr ***	
	tyr trp ile gly ile arg lys val glu gly val trp thr trp val gly	64
	*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***	
	*** *** *** *** *** *** *** ile gly *** ile *** *** *** *** ***	
	*** *** *** *** *** *** *** ile gly lys met *** *** *** *** ***	
	thr asn lys ser leu thr glu glu ala lys asn trp gly ala gly glu	80
	*** *** *** *** *** *** *** *** *** *** *** *** *** glu *** ***	
	*** *** *** *** *** *** *** *** *** glu *** *** *** asp *** ***	
	*** *** *** thr *** *** lys *** *** glu *** *** *** asn *** ***	
	pro asn asn arg lys ser lys glu asp cys val glu ile tyr ile lys	96
	*** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***	
	*** *** *** lys *** asn *** *** *** *** *** *** *** *** *** ***	
	*** *** *** lys *** *** *** *** *** *** *** *** *** *** *** ***	
	arg asn lys asp ser gly lys trp asn asp asp ala cys his lys ala	112
	*** ile *** *** *** *** *** *** *** *** *** *** *** *** *** ***	
	*** *** *** *** ala *** *** *** *** *** *** *** *** *** *** leu	
	*** glu arg *** *** *** *** *** *** *** *** *** *** *** *** arg	
	lys lys XX	114
	*** ***	
	*** ala	
	*** ala	

Figure 6. Comparison of the bovine and sheep L-selectin lectin domain predicted amino acid sequence (based upon the cDNA sequence) to the human and mouse L-selectin homologues. The bovine and sheep lectin domain amino acid sequence begins with Trp residue number one which represents the probable NH_2-terminus for the mouse and human lectin domains. Potential N-glycosylation sites (Asn X Ser/Thr) are boxed at Asn positions 22 and 66 along with the additional site unique to the bovine and sheep at Asn position 39. The bovine lectin domain amino acid sequence displays a high degree of identity to the human and murine L-selectin lectin domains, 81.6% and 76.3% respectively. The last two COOH-terminal amino acids (XX) were included within the PCR extension primer and were not listed for the derived amino acid sequence.

reactivity was seen in sheep and goats (data not shown). In sites of chronic inflammation (inflamed synovitis) DREG-200 and DREG-152 reacted with vascular endothelium (Figure 8).

Discussion

The value of larger domestic animals in studies of lymphocyte trafficking mechanisms has long been appreciated. The ease of cannulation techniques, the ability to harvest large numbers of cells, and the exquisite tissue-selective homing properties of lymphocytes taken from specific sites in the animal are some of the reasons many of the paradigms of lymphocyte homing are based upon studies in these large animals (1,2, 12-17). Recently, models have been established for LAD in cattle and γ/δ T-cells in both cattle and sheep (22,23). Though much is known concerning the biology of leukocyte homing in large animals, few of the molecular mechanisms that control these processes have been defined. This is an important goal if the large animal models are to be exploited for molecular immunological studies.

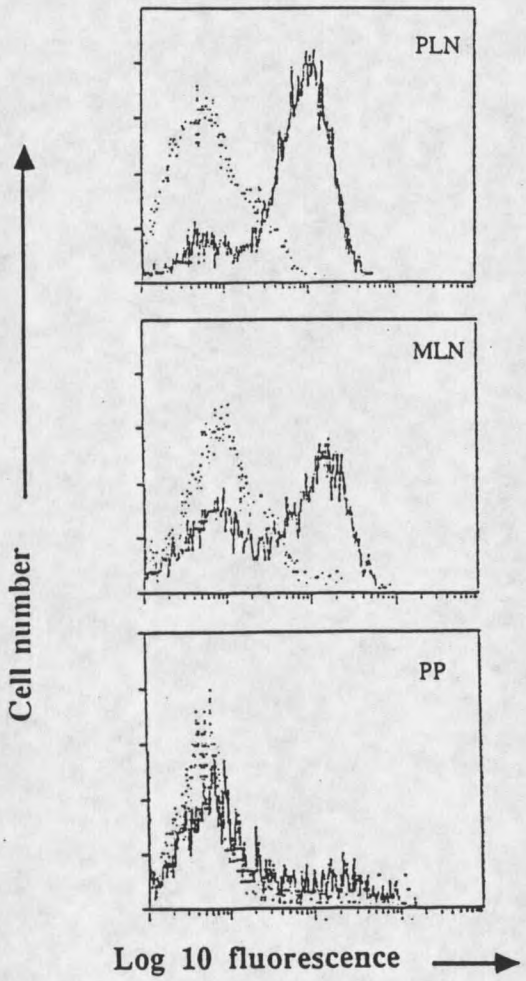

Figure 7. L-selectin expression on lymphocytes isolated from peripheral and MLNs, and Peyer's patches. Cells were isolated from the indicated tissues and stained with DREG-56 followed by FITC-anti-mouse Ig second stage. The solid line indicates the staining of DREG-56 and the dotted line reflects the staining of the negative control antibody. PLN, peripheral lymph node lymphocytes; MLN, mesenteric lymph node lymphocytes; and PP, Peyer's patch lymphocytes.

By examining its molecular nature as well as its function and regulation, we found that bovine L-selectin is remarkably similar to the human and mouse molecules. Four anti-L-selectin antibodies (DREG-56, DREG-200, DREG-152, and Leu-8) crossreact in the cow. The bovine homing receptor is regulated much like the human and mouse molecule, and

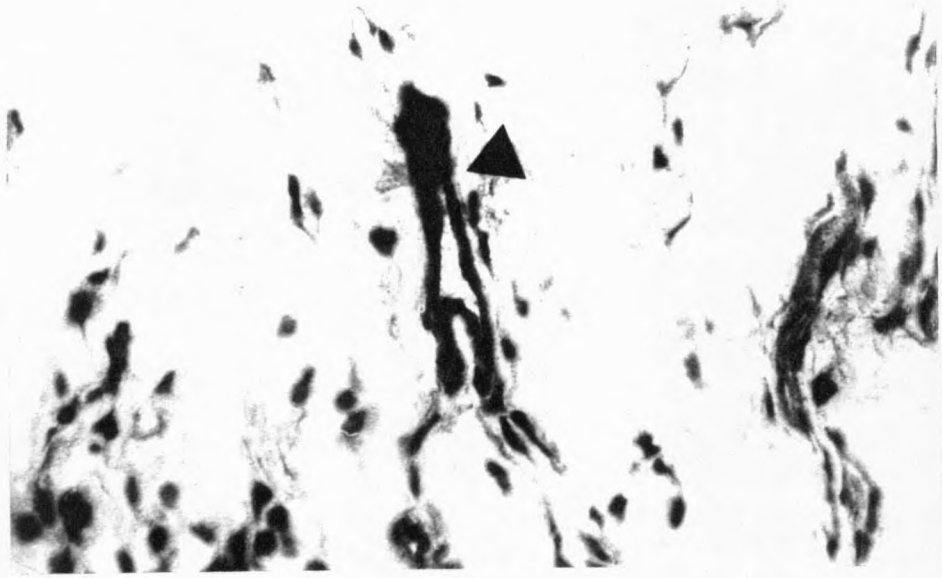

Figure 8. Anti-human L-selectin antibodies stained venules in chronically inflamed synovitis. Frozen sections of inflamed synovium were prepared and stained with DREG-200 mAb for immunohistology, as described in Materials and Methods. The arrow points to a positive staining venule. Magnification approximately 400X.

anti-L-selectin mAbs block bovine lymphocyte adhesion to PLN HEV. Limited sequence analysis of the bovine L-selectin lectin domain shows a very high level of identity at both the nucleotide and predicted amino acid level between the bovine, human, and mouse molecules. Bosworth and co-workers have used our lectin domain cDNA to clone the entire L-selectin cDNA and have found a similar level of identity throughout the whole protein. Therefore, at the molecular and functional level tremendous similarity is seen between the bovine, human, and mouse L-selectin, indicating a high level of evolutionary conservation of this family of adhesion proteins. The conservation of L-selectin between such diverse mammalian species points to the obvious importance of these molecules to the survival of the host.

Though bovine L-selectin is very similar to the human and mouse homologues, there are interesting differences. For instance, sequence analysis demonstrates an additional N-

linked glycosylation site in both the bovine and sheep L-selectin lectin domain. What difference this may convey to the function or regulation of the molecule is not known. Interestingly, even though there is >96% identity between the sheep and cow L-selectin lectin domain, the mAbs DREG-56, -152, -200 and Leu-8 do not stain sheep L-selectin. A much greater separation of L-selectin expression in bovine lymphoid tissues exists versus what has been described in the mouse. Most cells in bovine Peyer's patch and the lamina propria are L-selectin negative, whereas, >80% of the cells in the PLNs are L-selectin positive. The percentage of L-selectin positive cells in MLNs is intermediate. It has been suggested that the primary site of B-cell generation in ruminants is the ileal Peyer's patch (42,43). If this is true, it will be interesting to determine at what point in the developmental stage B-cells express L-selectin, which to date has only been described for the T-cell. The tissue-restricted expression of bovine L-selectin is also consistent with the observations concerning the tissue-selective homing properties of lymphocytes from different lymphoid tissues of ruminants (16). Finally, two anti-human L-selectin antibodies, which recognize bovine L-selectin, react with endothelial cells of cow, goat, and sheep. Of particular interest is the staining of endothelial cells in chronically inflamed synovium, whereas in most other tissues the staining is restricted to arterial smooth muscle and granules within endothelial cells. Recently, we have found that cultured bovine and sheep endothelial cells produce antigens recognized by these antibodies (data not shown). Endothelial cells express surface proteins that are very similar to leukocyte L-selectin. For example, P-selectin and E-selectin are expressed by inflamed endothelium and exhibit nucleotide sequence similarities of >60% with L-selectin (8,9). We are currently determining if the endothelial cell antigen recognized by DREG-200 and DREG-152 is one of these two selectins or represents a new member of this family of adhesion proteins.

In summary, we have provided an extensive characterization of bovine L-selectin. Considerable similarities are seen between the bovine molecule with homologous proteins in the human and mouse, yet notable differences exist. Our results establish tools for exploiting the cow as a useful model for leukocyte trafficking studies, and provide additional impetus for using larger animals for the molecular characterization of other adhesion systems, including other homing receptors and even the vascular addressins (48,49).

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CHAPTER 3

BOVINE γ/δ T CELLS EXPRESS HIGH LEVELS OF FUNCTIONAL PERIPHERAL LYMPH NODE HOMING RECEPTOR (L-SELECTIN)

Introduction

Lymphocytes recirculate from the blood through various tissues, which enables their diverse antigenic specificities to be continually represented throughout the body (1). Unlike α/β T cells, γ/δ T cells do not constitutively traffic in large numbers through secondary lymphoid tissues; but instead exhibit a homing preference for epithelial cell-associated tissues, such as the skin, lungs, gut, mammary glands, and tongue (2,3).

The process of lymphocyte extravasation from the blood into the underlying tissue involves at least two distinct adhesive mechanisms (1,4). The first involves tissue-selective receptor/ligand pairs between lymphocytes and the endothelial cell lining of post-capillary venules (PCV). The lymphocyte and endothelial cell tissue-selective adhesion molecules are referred to as homing receptors and vascular addressins, respectively (1). The second adhesive mechanism involves accessory adhesion molecules, such as the integrins and CD44, which control transendothelial migration.

The most thoroughly characterized lymphocyte homing receptor is L-selectin, which is an 80 kDa glycoprotein that mediates adhesion to the peripheral lymph node (PLN) vascular

addressin (MECA-79 antigen) (5-9). Previous studies have demonstrated that leukocytes rapidly downregulate L-selectin expression following PMA activation (8,10-12), which is believed to occur via a chymotrypsin-like, membrane-associated protease (13). The rapid downregulation of L-selectin may serve an important short-term role in regulating lymphocyte extravasation by releasing the lymphocyte from its primary adhesive bonds and allowing it to continue migration through secondary adhesive events.

Expression of L-selectin has been proposed to be prognostic of the ability of lymphocytes to recirculate through PLN. For instance, all lymphocytes that recirculate through PLN are L-selectin positive. Cells that lack L-selectin expression do not home through PLN, but exhibit preferences for other sites (14,15). Similarly, γ/δ T cells may represent another lymphocyte population whose lack of homing to PLN is due to the absence of functional L-selectin expression, but this has not been directly tested.

The study of γ/δ T cells in humans and mice is problematic, since these cells represent a minor population of the T cells in peripheral blood (typically <5%) (16). Ruminants contain a substantial fraction of these cells in their circulatory system: in the immature animal, the γ/δ T cell population may represent up to 50% of the peripheral blood mononuclear cells, typically declining to 10-25% as the animal matures (17). Much of what is known about the biology of γ/δ T cells has come from studies of sheep and cattle (3). Cattle possess three prominent T cell populations: 1) $CD2^+$ $CD4^+$ $CD8^-$, 2) $CD2^+$ $CD4^-$ $CD8^+$, and 3) $CD2^-$ $CD4^-$ $CD8^-$ (null cells) (18). The CD2 lineage marker denotes α/β T cells. The bovine null cells predominantly represent γ/δ T cells. These cells can also be identified by a lineage-specific marker known as the Workshop Cluster 1 antigen [(WC1) also referred to as T19], a 165/275 kDa cell surface molecule (3,19-21).

In this study, we use the cow to examine whether the lack of γ/δ T cell homing to PLN is due to deficient L-selectin expression or function. We show that peripheral blood γ/δ T

cells actually express higher levels of L-selectin than other lymphocyte subsets and adhere to PLN PCV in vitro and in vivo, but they do not extravasate into the underlying tissue. Thus, events subsequent to primary adhesion must also contribute to the selectivity of lymphocyte homing. Indeed, though the γ/δ T cells appear to express the necessary secondary adhesion proteins for extravasation, they do not regulate L-selectin expression under short-term activation conditions in the same manner as α/β T cells.

Materials and Methods

Animals

Mixed breed cattle ranging in age from <1 month to >1 year were purchased from local producers and housed at the Montana State University large animal facilities in the Veterinary Molecular Biology Laboratory. Peripheral blood was collected into heparinized tubes by venipuncture of the jugular, and the mononuclear cells were separated by centrifugation through ficoll-hypaque (Histopaque 1083, Sigma, St Louis). Tissue samples were collected from untreated animals upon necropsy. All mice used were BALB/c females ranging in age from 6-12 weeks and housed at the Montana State University small animal facility.

Reagents

The mAbs used in this report have been described elsewhere. DREG-56 and DREG-200 are mouse mAbs that recognize human (8) and bovine (22) L-selectin. All L-selectin staining results presented in this paper were repeated with other mAbs that also recognize bovine L-selectin, such as Leu-8 (22), EL-246 (23), and DREG-152 (22) (data not

shown). IL-A29 (ATCC, Rockville, MD) and BAQ4A (VMRD, Pullman, WA) are mouse mAbs that recognize the 165/275 kDa WC1 antigen specifically expressed by bovine, ovine, and caprine γ/δ T lymphocytes (24). CC42 and CC21 (both generously provided by Chris Howard, Institute for Animal Health, U.K.) are mouse mAbs that recognize CD2 and a 145 kDa B-cell marker, respectively, on bovine and caprine lymphocytes (24). Hermes 3 (25-27) is a mouse mAb that recognizes CD44 (HCAM) on human leukocytes and cross-reacts with bovine lymphocytes (22). 60.3 (generously provided by John Harlan, University of Washington) is a mouse mAb that recognizes CD18 on human leukocytes (28) and crossreacts with ruminant leukocytes. MECA-79 (generously provided by Eugene Butcher, Stanford University) is a rat mAb that recognizes the human PLN vascular addressin (29) and cross-reacts with bovine and ovine PCV endothelial cells within the PLN (30, Jutila, M.A. unpublished observation).

Two-color immunoperoxidase staining

Bovine PLN (prescapular and subiliac lymph nodes collected from animals <3 months of age) were sectioned at 6 um, fixed in acetone and blocked with 10-15% rabbit serum for 20 minutes. First stage antibody (mAb #1, MECA-79) in PBS (50ug/ml) or undiluted culture supernatant was added to the sections and allowed to incubate for 30 minutes at room temperature in a humidified chamber. Sections were washed in PBS and incubated with second stage biotinylated anti-rat antibody (Histoprobe, Tago, Burlingham, CA). After another PBS wash, the sections were incubated with streptavidin peroxidase (Histoprobe, Tago, Burlingham, CA) for 20 minutes. The MECA-79 mAb was revealed with the substrate 4-chloro-naphthol (Sigma, St. Louis, MO) which gave a blue color. The process was then repeated with IL-A29 (mAb #2, anti-γ/δ T cell) which was revealed by second stage biotinylated anti-mouse antibody (Histoprobe, Tago, Burlingham, CA),

streptavidin peroxidase, and amino-ethyl carbazol (Sigma, St. Louis, MO) which gave a red color. Tissue sections were also incubated with isotype-matched negative control mAbs to determine any nonspecific binding of antibodies and/or endogenous peroxidase development of the substrates.

Immunofluorescence staining and fluorescent activated cell sorting (FACS) analysis

The immunofluorescence staining procedure was carried out in 4 ml tubes (Becton Dickinson, Lincoln Park, NJ). Typically, $1x10^6$ cells per tube were initially incubated in 2% rabbit serum for 10 minutes on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 ug/ml (or undiluted culture supernatant) for 20 minutes on ice. After washing, bound antibodies were revealed by incubation with PE conjugated F(ab)'2 goat anti-mouse Ig (Histoprobe, Tago, Burlingham, CA). FACS analysis was performed on a FACScan (Becton and Dickinson, Mountain View, CA), as previously described (10,13,31). In all analyses, lymphocytes were identified by their unique forward and side light scatters. For two-color analysis, FITC-conjugated DREG-56 and IL-A29 were used after staining with the primary mAb. Before cells were stained with the FITC-conjugated mAb, they were treated with 10% mouse serum to block any available anti-mouse Ig binding sites of the second stage. Data were collected from 10,000-50,000 cells and presented as contour plots. Background fluorescence was determined by an isotype matched negative control mAb. Nonspecific staining by dead cells was determined by the uptake of propidium iodide (Sigma, St. Louis, MO) and subsequently negated from FACS plots and quantitative values.

In vitro PMA and chymotrypsin treatment of bovine peripheral blood lymphocytes (PBL)

Isolated bovine PBL (from animals <3 months of age) were treated with phorbol 12-myristrate 13-actetate (PMA) (Sigma, St. Louis, MO) (50-100 ng/ml) for 2-30 minutes at 37°C in HBSS (JRH Biosciences, Lenexa, KS). After the incubation time, cells were fixed in a 0.5% final concentration of paraformaldehyde (Sigma, St. Louis, MO), washed, and then stained for FACS analysis. The effects of chymotrypsin (Sigma, St. Louis, MO) were tested as previously described (13).

Ex vivo high endothelial cell venule (HEV) assay

The ex vivo assay of lymphocyte binding to HEV in frozen sections has been extensively described (1,8,22,32-35). No modifications were done to measure bovine lymphocyte-HEV interactions.

In vivo homing assay

Bovine PBL (from animals <1 month of age) were resuspended in HB101 serum-free media (Irvine Scientific, Santa Ana, CA) and FITC labeled, as previously described (13). Media containing serum caused agglutination of the leukocytes. Three $x10^7$-$5x10^7$ FITC-labeled cells in a 0.5 ml volume were injected iv into 6-12 week old BALB/c mice. A small volume of cells were retained for determination of initial percentages of α/β T, γ/δ T, and B lymphocytes (ascertained by FACS analysis using lineage-specific mAbs). The injected cells were allowed to home for 3-4 hours, after which peripheral blood was collected, the mice sacrificed, and cell suspensions made of the thymus and secondary lymphoid organs. FACS analysis was done to quantify the level of lymphocyte entry into the various organs. Thymus was included as an internal negative control due to the inability of mature lymphocytes to return to this organ. Using lineage-specific mAbs with PE-labeled second

stage, the migration of distinct lymphocyte subsets into a particular lymphoid organ was measured.

B-cell panning

In order to compare CD44 and CD18 expression between γ/δ and α/β T cells, B-cells were removed from the lymphocyte population to leave a predominant T cell population. Bovine peripheral blood was collected and mononuclear cells were separated by centrifugation through Histopaque 1083. Adherent cells were removed by incubating the mononuclear cells in a Falcon polystyrene flask (Becton Dickinson, Plymoth, England) for one hour at 37°C, 10% CO_2. The resulting nonadherent population, at an initial concentration of $5x10^7$ cells, was sequentially passed over 2 plates precoated with an anti-B-cell mAb (CC21). The resulting negatively selected population was stained with mAbs and analysed by FACS. The gated lymphocyte population contained <2% $CC21^+$ B-cells and up to 90% T cells.

Results

Bovine γ/δ T cells express L-selectin

In separate reports, we showed that the DREG-56, DREG-152, DREG-200, EL-246 and Leu-8 anti-human L-selectin mAbs stain bovine leukocytes (22,23). Bovine L-selectin was regulated like the human homologue, and DREG-56 specifically blocked bovine lymphocyte adhesion to PLN HEV. Additionally, the nucleotide and predicted amino acid sequence of bovine L-selectin were greater than 80% identical to the human molecule (22). Therefore, at the functional, biochemical, and molecular level, bovine L-selectin is

homologous to human L-selectin.

Two-color FACS analysis using mAbs that specifically recognize the WC1 lineage marker on bovine γ/δ T cells (IL-A29 and BAQ4) versus FITC-labeled DREG-56 was done to determine the level of L-selectin expression on γ/δ T cells. We found that essentially 100% of the γ/δ T cells in immature animals (<3 months of age) expressed L-selectin. Interestingly, the level of L-selectin expression on γ/δ T cells was consistently 2-5x greater than that seen on other circulating lymphocytes in the same animal (>10 animals examined). Figure 9A and B show representative FACS plots of the two-color stain. To verify that this increase in DREG-56 staining was not due to inefficient compensation within the FL2 channel (PE) during the two-color analysis, we also stained the γ/δ T cells with FITC-labeled IL-A29 versus DREG-56, which gave similar results (Figure 9C). Figures 9D and E show the level of L-selectin expression on α/β T- (mode fluorescence = 40) and B lymphocytes (mode fluorescence = 13), respectively, demonstrating that these populations were L-selectin moderate compared to the L-selectin bright γ/δ T cells (mode fluorescence = 107). In the mature animal (>6 months of age), peripheral blood γ/δ T cells, which were reduced in numbers, remained essentially 100% L-selectin positive, compared to non-γ/δ lymphocytes which demonstrated a bimodal distribution of L-selectin expression (Figure 10). Furthermore, L-selectin expression remained higher on γ/δ T cells than non-γ/δ lymphocytes (Figure 10).

γ/δ T cells adhere to PLN PCV

Though γ/δ T cells express high levels of L-selectin, the expressed form may be nonfunctional, which could account for their lack of migration from the blood into PLN. Using two-color immunoperoxidase analysis we examined if γ/δ T cells adhered to PLN PCV in vivo. Multiple frozen sections of bovine PLN from several different animals were

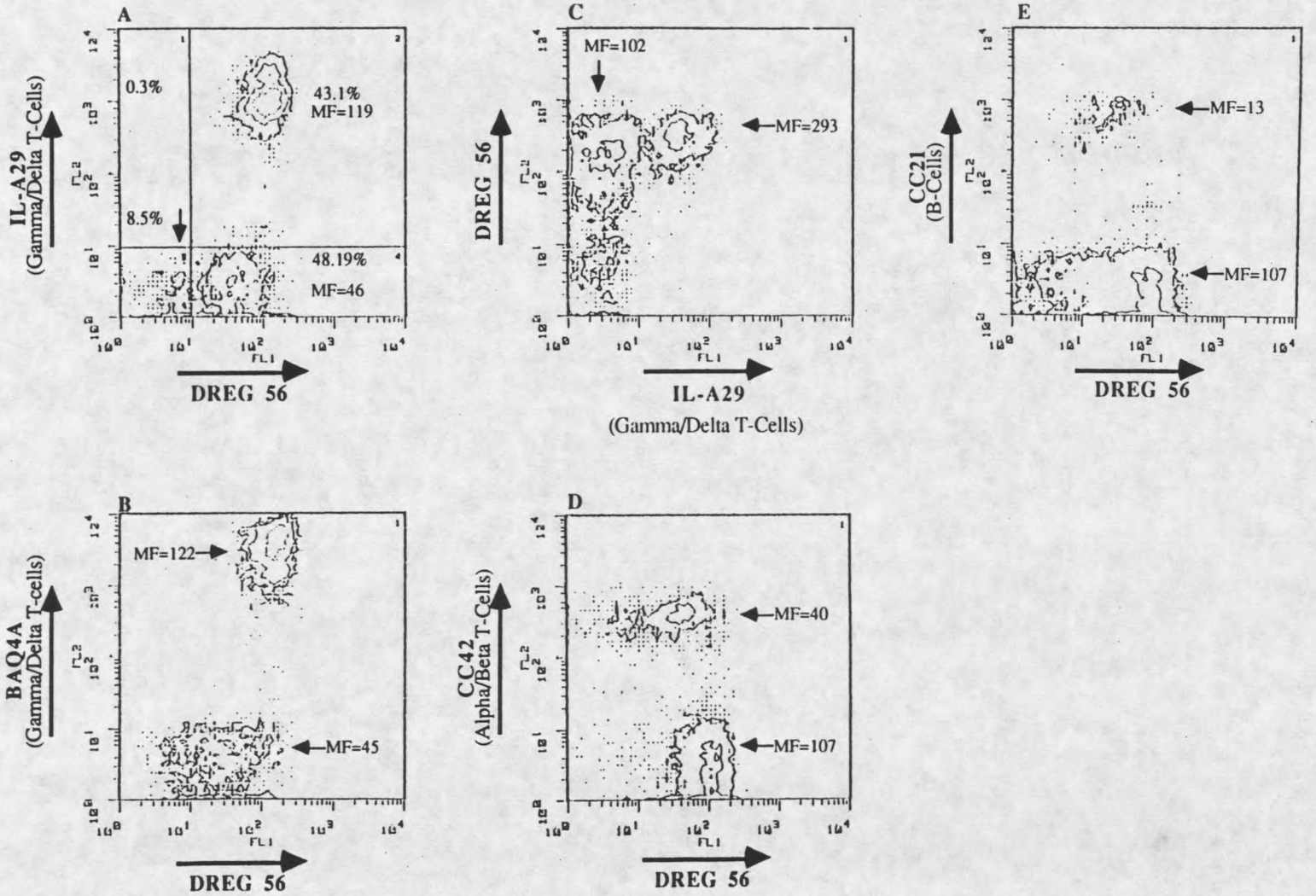
A
0.3%
43.1%
MF=119
8.5%
48.19%
MF=46
IL-A29
(Gamma/Delta T-Cells)
DREG 56
B
MF=122
MF=45
BAQ4A
(Gamma/Delta T-cells)
DREG 56
C
MF=102
MF=293
DREG 56
IL-A29
(Gamma/Delta T-Cells)
D
MF=40
MF=107
CC42
(Alpha/Beta T-Cells)
DREG 56
E
MF=13
MF=107
CC21
(B-Cells)
DREG 56

Figure 9. L-selectin expression on peripheral blood lymphocyte subsets isolated from immature animals (<3 months of age). Cells were isolated from peripheral blood and stained for two-color FACS analysis with DREG-56 (anti-L-selectin) and lymphocyte lineage-specific mAbs, as described in Materials and Methods. The level of L-selectin expression is represented by mode fluorescence (MF) of positive staining cells. Panel A shows the level of L-selectin expression on γ/δ T cells (IL-A29 bright, quadrant 2) compared to non-γ/δ lymphocytes (quadrant 4). Panel B shows similar staining results as in panel A using another anti-γ/δ T cell mAb, BAQ4A.; Panel C shows the level of L-selectin expression on γ/δ T cells using FITC-labeled IL-A29 vs DREG-56; and panels D and E show moderate levels of L-selectin expression on α/β T cells (CC42) and B-cells (CC21), respectively, compared to the L-selectin bright γ/δ T cells. Background fluorescence with an isotype control was subtracted out of percentages and MF of positive-staining cells.

prepared, fixed in acetone, and stained with the anti-vascular addressin mAb MECA-79 and a γ/δ T cell-specific mAb IL-A29. We found that virtually all MECA-79-positive venules contained γ/δ T cells bound to their luminal surface. However, the percentage of γ/δ T cells in the underlying parenchymal tissue was <5% [confirmed by FACS analysis of bovine PLN cell suspensions as well (data not shown)]. Figure 11 shows an example of a two-color immunoperoxidase stain in which a MECA-79-positive venule contains several γ/δ T cells associated with the stained vascular endothelium, but few cells in the parenchymal tissue.

Using the Stamper and Woodruff ex vivo adhesion assay, which measures homing receptor-dependent adhesion of lymphocytes to lymphoid HEV (32), it has been shown that many of the tissue-selective adhesion molecules are functionally conserved between diverse animals (36,37). We (22) and others (34,35) have shown that ruminant peripheral blood lymphocytes bind mouse PLN HEV, and the interaction is inhibited by an anti-L-selectin mAb. Using this assay we examined whether γ/δ T cells represented a portion of the HEV-bound lymphocytes. We incubated bovine PBL with mouse PLN sections as previously described (22), and stained the cells bound to the HEV for γ/δ T cells by immunoperoxidase staining using biotin-labeled IL-A29. Shown in figure 12 is a

representative binding assay with both γ/δ T cells and non-γ/δ lymphocytes bound to a HEV.

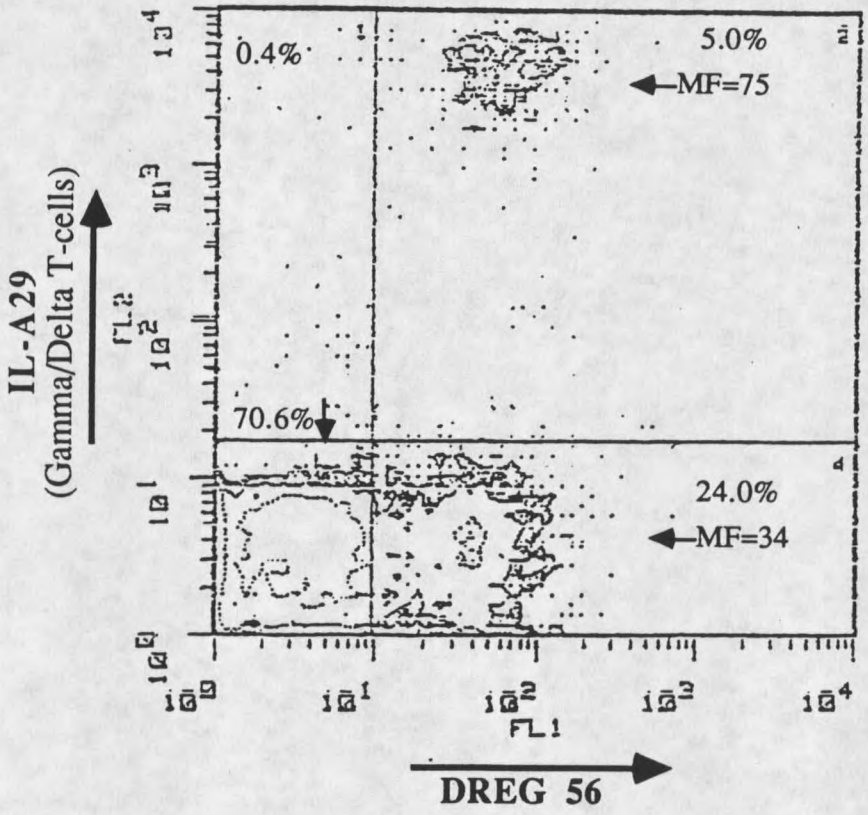

Figure 10. L-selectin expression on peripheral blood lymphocyte subsets isolated from mature animals (>6 months of age). Cells were isolated and stained for L-selectin expression (FITC-labeled DREG-56) and γ/δ T cells (IL-A29), as described in figure 9. Background fluorescence with an isotype control was subtracted out of percentages and MF of cells staining positive.

Lymphocytes from diverse animals not only bind mouse HEV, but in a separate study we found they actually accumulate in a tissue-selective manner within the mouse lymphoid organs in short-term in vivo homing assays. For example, lymphocytes isolated from the mucosal tissue of sheep, goat, or cow preferentially home to mucosal tissues when injected into mice (Bargatze, R.F. and Jutila, M.A. manuscript in preparation). Furthermore,

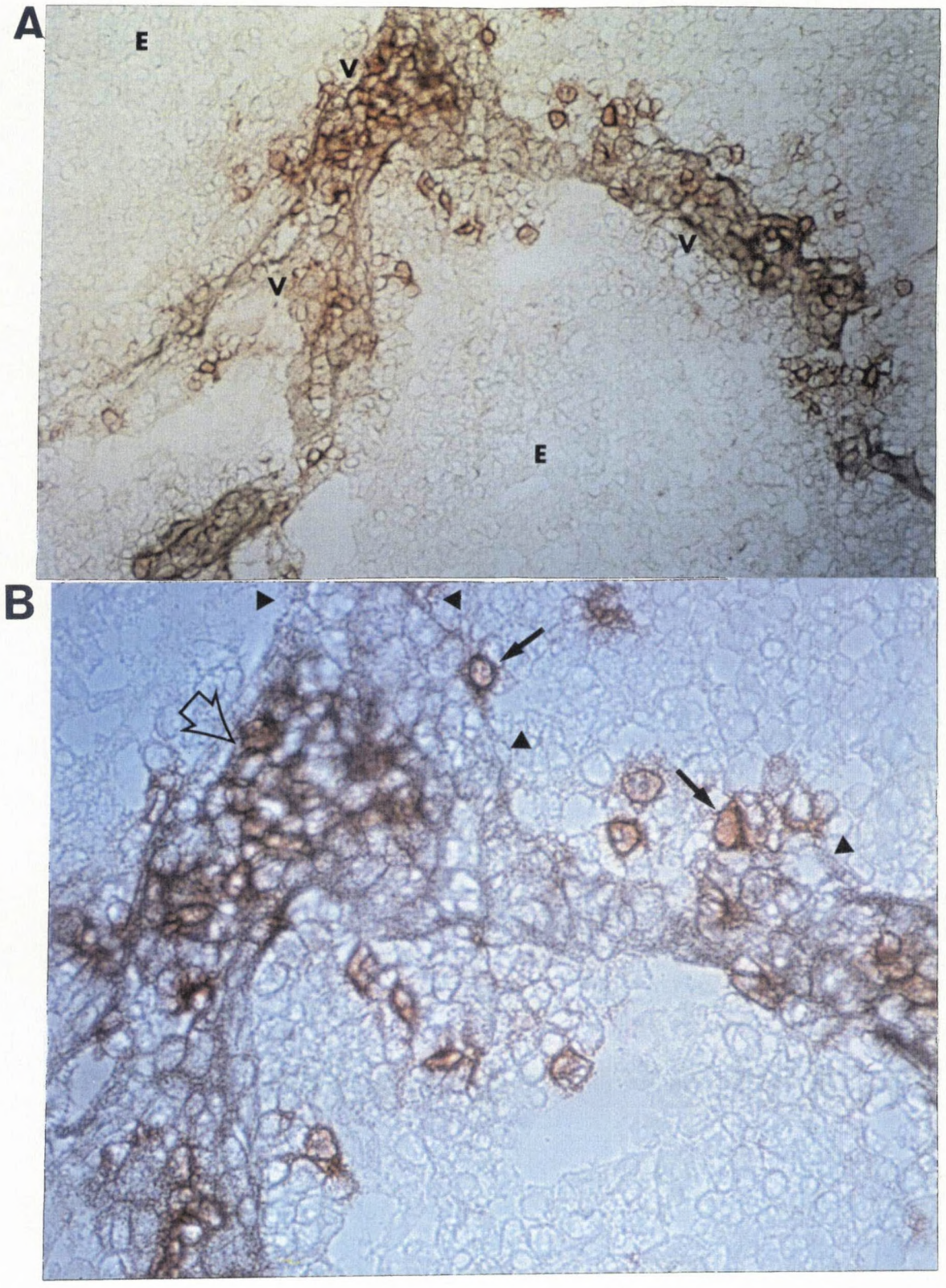
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Figure 11. γ/δ T cells adhere to the vascular wall of MECA-79-positive PLN PCV. Frozen sections of calf PLN were prepared and stained with MECA-79 (anti-PLN vascular addressin) and IL-A29 (anti-γ/δ T cell), as described in Materials and Methods. Figure 11A shows a large MECA-79-positive venule (V) that contains several γ/δ T cells associated with its vascular wall, but few cells in the extravascular tissue (E) (magnification approximately 400X). Figure 11B shows a region from figure 11A at increased magnification (approximately 600X). The arrows ( ) detail the outline of a portion of the MECA-79-positive venule wall in which the γ/δ T cells can be seen ( ). The open arrow points to a large accumulation of γ/δ T cells in the venule which occurs throughout the width of the section, producing a blurred image.

bovine PBL readily accumulate in mouse PLN, which can be blocked by anti-L-selectin mAbs (Jutila, M.A. et al. manuscript submitted).

Using the xenogeneic assay and two-color flow cytometry, we compared the capacity of bovine peripheral blood α/β and γ/δ T cells to enter mouse PLN. FITC-labeled bovine PBL were injected iv into 6-12 week old BALB/c mice and allowed to home for 3-4 hours. Figure 13 shows a representative experiment in which there were 1.1% $FITC^+$ cells in the PLN, 1.7% $FITC^+$ cells in the mesenteric lymph nodes, 0.95% $FITC^+$ cells in the Peyer's patch, 5.1% $FITC^+$ cells in the spleen, 1.1% $FITC^+$ cells in the peripheral blood, and 0% $FITC^+$ cells in the thymus following the homing assay. These results are similar to the homing of syngeneic mouse lymphocytes which make up 2-3% of a lymphoid organ's population following injection of an equivalent number of cells (Bargatze, R.F. and Jutila, M.A. unpublished observation). Using lineage-specific mAbs, we measured the migration of lymphocyte subsets by two-color FACS analysis. Summarized in table 4 are the initial percentages of α/β T-, γ/δ T-, and B-lymphocytes found in bovine peripheral blood, and the percentages of each subset from the total number of FITC-labeled cells that migrated into a particular mouse lymphoid organ. γ/δ T cells, unlike α/β T cells, did not significantly accumulate in any of the secondary lymphoid sites, except for spleen (Table 4), though these cells did accumulate in large numbers in mouse intestinal lamina propria revealed by

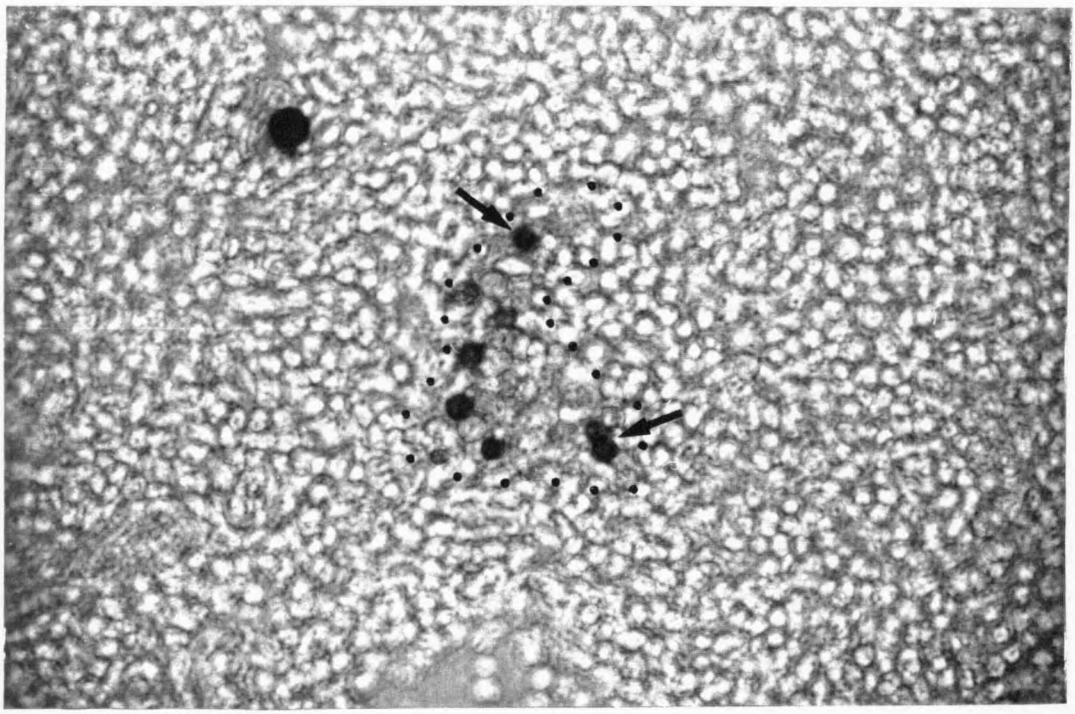

Figure 12. Bovine γ/δ T cell adhesion to mouse PLN HEV. The ex vivo HEV binding assay was performed as described in Materials and Methods. γ/δ T cells (arrow) were revealed by immunoperoxidase staining with biotin-labeled IL-A29. All HEV (dotted outline) bound lymphocytes were not in the same plane of focus, producing a blurred image of certain cells.

immunoperoxidase staining (Figure 14). These results are consistent with what has been described for γ/δ T cell homing in the ruminant (3). The lack of γ/δ T cell extravasation into PLN was not due to xenogeneic differences in the mechanisms controlling this event since α/β T cells efficiently entered this site.

γ/δ T cell L-selectin is not downregulated as efficiently as L-selectin on α/β T cells

It is well established that L-selectin is rapidly downregulated on lymphocytes (within 30 minutes) after treatment with PMA (8,11,12). We examined by two-color FACS analysis whether γ/δ T cells downregulate L-selectin expression in response to PMA the same as α/β

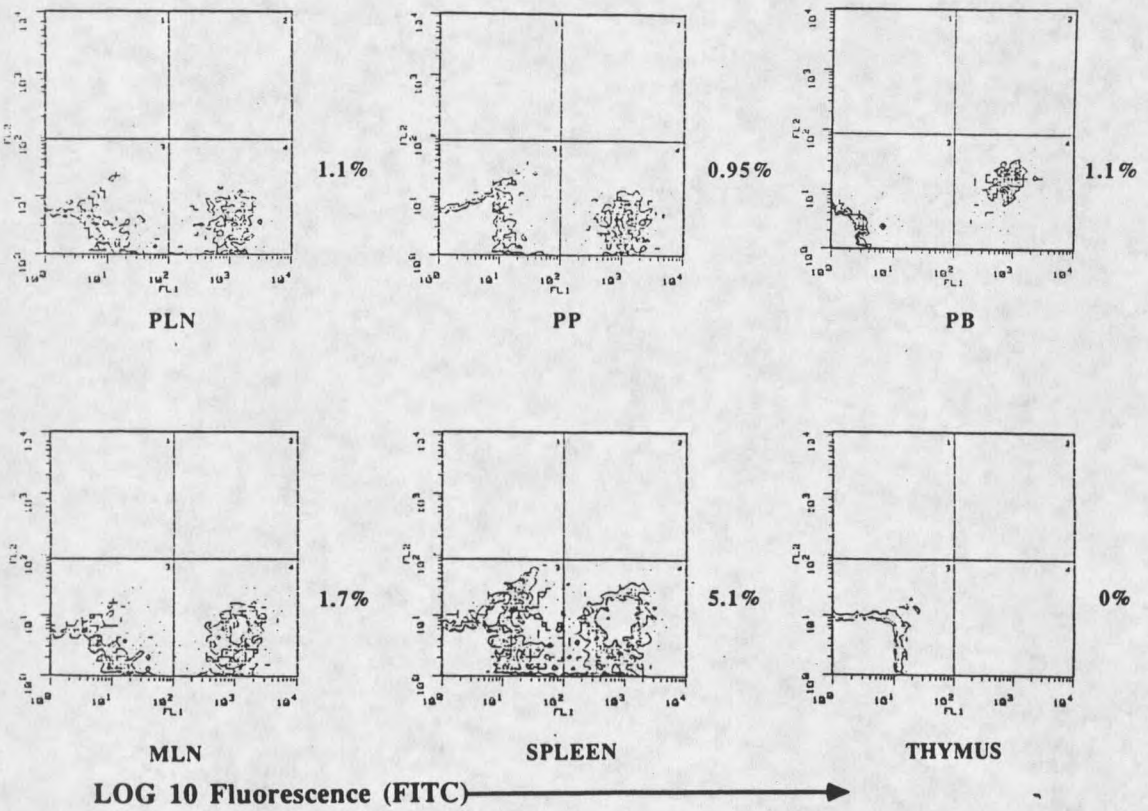

Figure 13. Short-term in vivo homing of FITC-labeled bovine peripheral blood lymphocytes to mouse PLN, mesenteric lymph nodes (MLN), Peyer's patch (PP), spleen, thymus, or cells remaining in the peripheral blood (PB). Bovine PBL were labeled with FITC and injected iv at a concentration of $3x10^7$-$5x10^7$ cells into a 6-12 week old BALB/c mouse, as described in Materials and Methods. The percentages of labeled cells versus total mouse lymphocytes in the respective lymphoid organs 3-4 hours postinjection were determined by FACS analysis of 50,000 cells and are represented as contour plots. The thymus was included as an internal negative control due to the inability of mature lymphocytes to return to this organ.

T cells. We found that in all kinetic experiments (5 animals examined) from 2-30 minutes after PMA treatment the γ/δ T cells expressed significantly higher levels of L-selectin at all time points compared to α/β T cells. Figure 15 shows a representative kinetic experiment in which after 30 minutes of PMA activation the mean fluorescence of L-selectin expression on α/β T cells was reduced from 61 to 22, whereas the mean fluorescence of L-selectin

Table 4. Migration of bovine PBL into mouse lymphoid organs following short-term in vivo homing.

	Percentages of bovine lymphocyte subsets				
	Peripheral blood [a]	Mouse lymphoid organs [b]			
		PLN	MLN	PP	spl[c]
γ/δ T cells	25.7+/-0.4	5.1+/-1.4	2.3+/-0.9	6.6+/-2.3	15.3
α/β T cells	45.8+/-3.9	86.3+/-2.4	90.4+/-1.1	80.1+/-5.2	63.3
B cells	8.1+/-1.9	6.1+/-5.2	1.9+/-0.8	2.5+/-0.8	1.8

a) Initial percentages of bovine lymphocyte subsets. Cells were isolated from peripheral blood and stained with lineage-specific mAbs for FACS analysis to determine the initial percentages of each lymphocyte subset. The FITC-labeled mononuclear cells were also stained with lineage-specific mAbs which showed that FITC labeling did not decrease the percentage of a particular lymphocyte subset (data not shown). The percentages listed represent the mean of three separate experiments +/- SEM. Background fluorescence with an isotype control was subtracted out of the percentages of positive-staining cells.
b) Percentages of bovine lymphocyte subsets from total FITC-labeled cells within the respective lymphoid organs. Short-term *in vivo* homing was performed as described in figure 13, after which the individual suspensions of each listed lymphoid organ were stained with lineage-specific mAbs and PE-labelled second stage, as described in Materials and Methods. The percentages listed represent the mean of three separate experiments +/- SEM. Background fluorescence with an isotype control was subtracted out of the percentages of positive-staining cells.
c) Spleen (Spl) percentages are of one experiment.

expression on γ/δ T cells was reduced from 298 to 133. Importantly, the percentage of γ/δ T cells remaining L-selectin positive following PMA activation for 30 minutes was 100% compared to 18% of the α/β T cells (Figure 15). Though γ/δ T cells did shed a certain

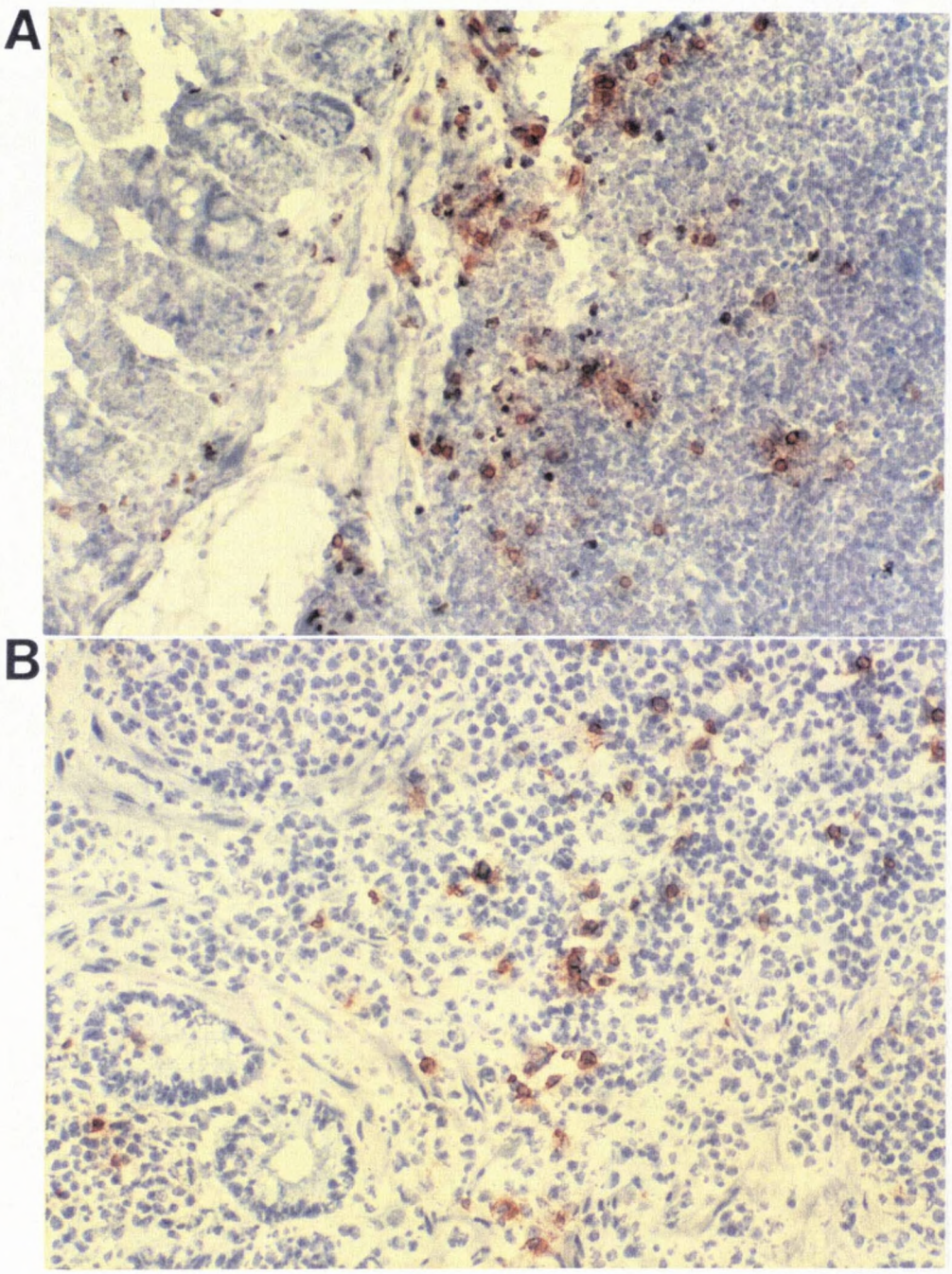

Figure 14. Bovine γ/δ T cells traffic to the intestinal lamina propria following short-term in vivo homing in the mouse. Bovine PBL were injected iv at a concentration of $3x10^7$-$5x10^7$ cells into a 6-12 week old BALB/c mouse. A) After 3-4 hours, the mice were sacrificed, intestinal regions collected, and sections were prepared for immunoperoxidase staining. The γ/δ T cells were revealed by biotin-labeled IL-A29. B) In comparison, an equivalent region of bovine intestines was stained for γ/δ T cells as well.

proportion of their L-selectin following activation, the level of expression remaining after 30 minutes was still >2X that of the α/β T cells at time 0 (Figure 15). Next we examined the rate of L-selectin downregulation on γ/δ T cells versus α/β T cells. PBL were treated with PMA over a time period of 2-10 minutes, and the percent decrease in L-selectin expression from time 0 was compared between γ/δ T cells and α/β T cells by two-color FACS analysis. We found that γ/δ T cells consistently downregulated their L-selectin at a rate slower than α/β T cells (Figure 16).

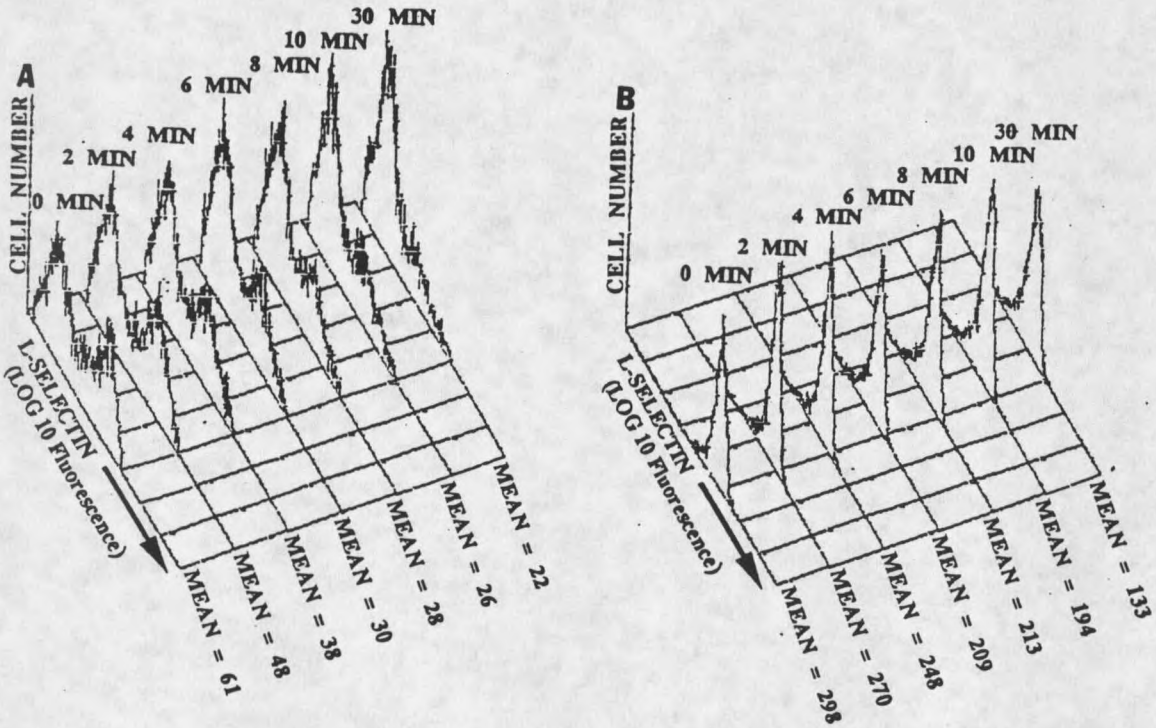

Figure 15. γ/δ T cells do not downregulate L-selectin to the level of expression on α/β T cells following PMA activation. Isolated PBL were treated with PMA (50-100 ng/ml) for 2-30 minutes, as described in Materials and Methods. The cells were then stained for two-color FACS analysis with FITC-labeled DREG-200 (anti-L-selectin) and (A) CC42 (anti-α/β T cells) or (B) IL-A29 (anti-γ/δ T cell). Background fluorescence with an isotype control was subtracted out of the MF of positive staining cells.

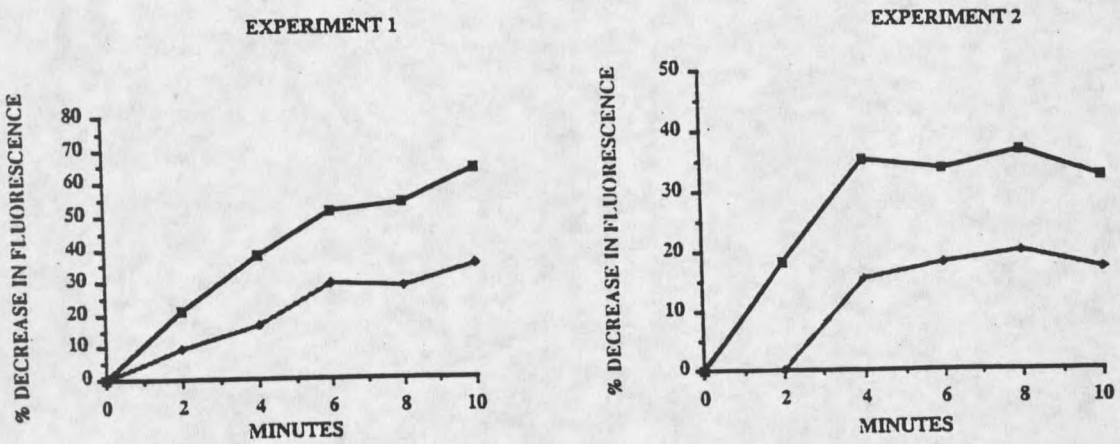

Figure 16. L-selectin downregulation occurs at a slower rate on γ/δ T cells (◆) compared to α/β T cells (■). Bovine PBL from two separate animals were treated with PMA for 2-10 minutes and stained for FACS analysis, as described in figure 15. The percent decrease in L-selectin expression from time 0 was determined by the formula: 100 - [L-selectin mean fluorescence (time n following PMA activation) / L-selectin mean fluorescence (time 0)].

Others have proposed that the downregulation of L-selectin on leukocytes after activation occurs via a unique proteolytic event (10,13). Recently, we have shown that low doses of chymotrypsin cause a specific release of neutrophil and lymphocyte L-selectin, which may potentially mimic the proteolytic process (13,22). We examined by two-color FACS analysis whether γ/δ T cell L-selectin possesses the same chymotrypsin-sensitive site as L-selectin on other lymphocytes. We treated PBL with chymotrypsin and stained for L-selectin expression on the γ/δ T cells. As shown in figure 17, the γ/δ T cells demonstrated an equivalent loss of L-selectin following chymotrypsin treatment compared to non-γ/δ lymphocytes (compare to figure 9, untreated cells).

γ/δ T cells express CD18 and CD44

We compared the expression of CD18 and CD44 on γ/δ and α/β T lymphocytes. First, B-cells were removed from a PBL preparation to leave predominantly α/β and γ/δ T cells

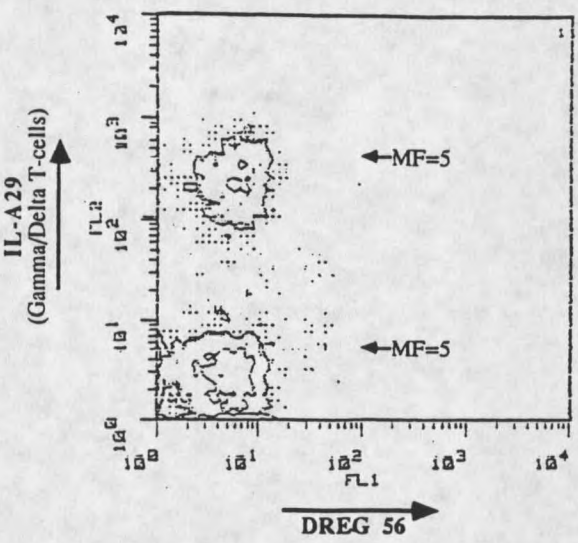

Figure 17. Low-dose chymotrypsin treatment of PBL results in an equivalent loss of L-selectin expression on γ/δ T cells and non-γ/δ lymphocytes. Isolated PBL were treated with 0.5U/ml of chymotrypsin for 15 minutes at 37°C. After protease treatment, the cells were fixed in 0.5% paraformaldehyde and stained for L-selectin expression (FITC-labeled DREG-56) and γ/δ T cells (IL-A29). Background fluorescence with an isotype control was subtracted out of the MF of positive-staining cells.

(approximately 90%), then two-color FACS analysis was performed using mAbs against CD44 or CD18 versus γ/δ T cells. Figure 18 shows the results from three separate animals. Peripheral blood γ/δ T lymphocytes expressed high levels of CD18 and CD44 which were equivalent to that seen on α/β T cells; however, on average, the γ/δ T cells did express slightly lower levels of CD44.

Discussion

T cell populations exhibit varied recirculatory patterns. Naive α/β T cells recirculate in a broad manner throughout all lymphoid tissues, whereas memory cells preferentially recirculate through specific lymphoid compartments (1). γ/δ T cells exhibit a homing

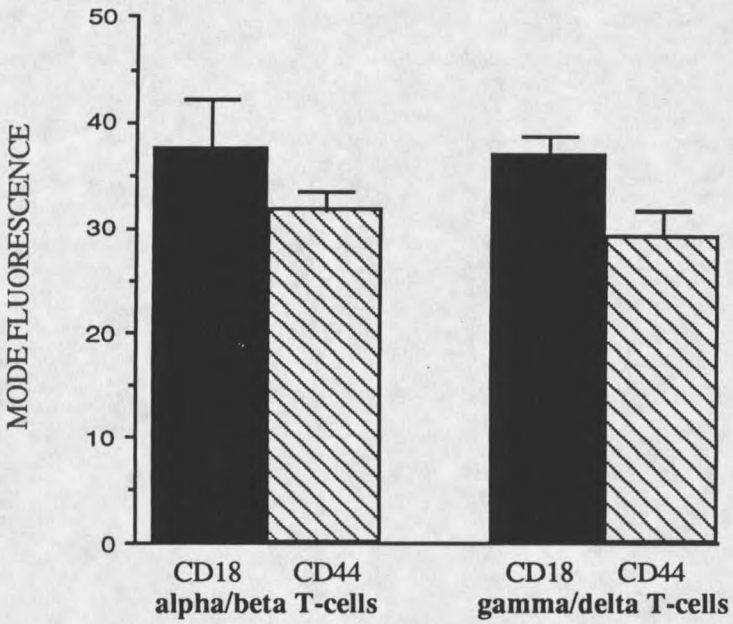

Figure 18. γ/δ and α/β T cells express equivalent levels of CD44 and CD18. B-cell depleted PBL were stained for two-color FACS analysis with anti-CD44 or anti-CD18 mAbs versus FITC-labeled IL-A29. Adhesion protein expression could be directly compared between IL-A29 negative lymphocytes (α/β T cells) and IL-A29 positive lymphocytes (γ/δ T cells). The MF values listed represent the mean from three separate animals tested +/- SEM. Background fluorescence with an isotype control was subtracted out of the MF of positive-staining cells.

preference to epithelial-associated tissues with few cells accumulating in secondary lymphoid sites, such as PLN (2,3). The basis for this lack of accumulation has not been determined. We tested whether bovine γ/δ T cells were deficient in L-selectin expression or function, since this adhesive mechanism is required for entry into PLN. We found that in young animals (<3 months of age) essentially all γ/δ T cells express L-selectin and at levels 2-5x greater than that seen on other peripheral blood lymphocytes. In mature animals (>6 months of age), variable numbers of α/β T cells were L-selectin positive, presumably due to the generation of memory lymphocytes which exhibit a characteristic bimodal expression

(38), whereas all of the γ/δ T cells remained L-selectin positive, though expression levels were less. γ/δ T cell L-selectin is functional as well, and the high level of expression allows for avid attachment of these cells to the vascular wall of PCV in PLN in vivo. In addition, γ/δ T cells appear to express the necessary secondary adhesion proteins, CD44 and CD18, for transendothelial cell migration, and at levels equivalent to α/β T cells. VLA-4 expression on γ/δ T cells was not examined due to the unavailability of mAbs that recognize the bovine homologue of this molecule. However, Issekutz T.B. has shown that a function-blocking mAb to VLA-4 does not affect the homing of PLN lymphocytes to PLN in vivo (39).

Since γ/δ T cells bind to PLN PCV that support lymphocyte entry, we used two separate approaches to confirm that these cells do not proceed with extravasation. First, using a novel xenogeneic homing assay, we found that bovine peripheral blood α/β T cells effectively migrate to lymphoid tissues in the mouse; however, γ/δ T cells do not, though they do adhere to the endothelial cell lining of mouse PLN HEV in vivo (data not shown). The short-term in vivo homing results are significant because they directly show that peripheral blood γ/δ T cells in the process of homing do not substantially accumulate within mouse secondary lymphoid organs via the blood, which is consistent to what has been previously shown by indirect methods in the ruminant (40-43). The γ/δ T cells were not considerably retained in mouse peripheral blood (data not shown) or spleen, suggesting that these cells are trafficking to other sites, such as the epithelial-associated tissues. In support of this, we have found that bovine γ/δ T cells accumulate in large numbers in mouse lamina propria (44). Our second approach consisted of two-color immunohistology. We found that γ/δ T cells adhere to MECA-79-positive venules, but were not found in significant numbers within the underlying tissue of PLN (<5%).

Our results are the first to show that expression of functional homing receptor on lymphocytes is not prognostic of the capacity of these cells to proceed with extravasation.

This situation has only been previously demonstrated for myeloid cells. Neutrophils express L-selectin, enabling them to adhere to mouse PLN HEV in the ex vivo assay (45); however, under normal conditions they are not found within the underlying tissue of lymph nodes. The study of neutrophils and γ/δ T cells demonstrates that there are multiple events that function in combination to ultimately control whether a leukocyte will enter a given tissue. In the case of the γ/δ T cell, we show that the inability to enter PLN correlates with their difference in regulation of L-selectin expression.

Rapid shedding of L-selectin in response to activating signals may allow the cell which is tightly bound to the vascular wall to more efficiently break those bonds and continue migration (8,11). Indeed, γ/δ T cells which avidly bind PLN PCV, but do not extravasate, differentially downregulate L-selectin following short-term PMA activation compared to α/β T cells. Previous studies examining the kinetics of lymphoid PCV extravasation by recirculating lymphocytes have demonstrated that this event occurs within a matter of minutes (46). Consequently, we directed our study of lymphocyte L-selectin downregulation by PMA activation to time points from 0 to 10 minutes. We found that at all time points (as well as at 30 minutes) γ/δ T cells retained significantly higher levels of L-selectin than α/β T cells. Furthermore, the γ/δ T cell's rate of L-selectin downregulation was consistently slower. The level of L-selectin downregulation following lymphocyte extravasation in PLN is unknown. This event may involve significant L-selectin shedding over the whole cell surface or focal loss at the site of lymphocyte/endothelial cell contact. Our previous studies suggest the latter may be the case (47). Regardless of focal or total lymphocyte L-selectin downregulation, the diminished capacity of γ/δ T cells to shed L-selectin at the rate and level that occurs on α/β T cells may contribute to their deficient extravasation into PLN and thus retain them on the luminal surface of PLN venules in vivo (Figure 11).

It was beyond the intent of this study to determine the molecular basis for the difference in L-selectin regulation on γ/δ T cells. However, it is likely that L-selectin is identical on γ/δ versus α/β T cells. γ/δ T cell L-selectin exhibits the same sensitivity to proteolysis and mediates the same binding interaction as L-selectin on other lymphocytes. In addition, our previous analysis of bovine lymphocyte L-selectin mRNA by PCR did not reveal differential transcripts (22). Therefore, the difference in regulation of L-selectin on α/β versus γ/δ T cells is likely due to differences in the activation/signalling pathways and/or expression of membrane-associated proteases involved in L-selectin shedding.

A recent report by Howard et al. (49) confirms our finding that essentially all peripheral blood γ/δ T cells in newborn and mature cattle are L-selectin positive. However, their report does not address function of γ/δ T cell L-selectin nor the inability of these cells to preferentially accumulate in PLN via the blood. In addition, the report states that bovine peripheral blood lymphocytes do not rapidly shed L-selectin following short-term treatment with PMA, which contrasts with our previous work (22) and current data for non-γ/δ lymphocytes (Figures 15 and 16), but is probably the result of inefficient L-selectin downregulation by γ/δ T cells.

In summary, to understand completely why γ/δ T lymphocytes traffic to epithelial-associated tissues, we must know why they do not enter secondary lymphoid sites. The ruminant provides an excellent model for the study of γ/δ T cell trafficking due, in part, to the large number of cells easily isolated from the blood. We show that inefficient γ/δ T cell homing to PLN compared to non-γ/δ lymphocytes is not due to a lack of L-selectin or its function. Preliminary results suggest that γ/δ and α/β T cells regulate their L-selectin differently. The γ/δ T cells reduced ability to downregulate L-selectin expression following short-term activation may distrupt the dynamics of the adhesive mechanisms involved in extravasation in PLN. Further study is obviously required to determine fully the molecular

mechanisms controlling the distinctive homing behavior of the γ/δ T cell.

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CHAPTER 4

BOVINE γ/δ T CELLS BIND E-SELECTIN VIA A NOVEL GLYCOPROTEIN RECEPTOR

Introduction

Selectins are a new family of adhesion proteins that direct leukocyte entry into sites of inflammation and lymphoid tissue (1). Two vascular (P- and E-selectin) and one leukocyte (L-selectin) selectin have been defined. From the predicted cDNA sequences, selectins are composed of an NH_2-terminal, C-type lectin domain followed by an epidermal growth factor domain, and multiple short consensus repeats (SCR) that are homologous to complement binding proteins. Selectins also have conventional transmembrane sequences and short cytoplasmic tails. The selectin lectin domains are required for function and their carbohydrate binding specificities have been partially defined. Sialylated Lewis carbohydrate derivatives, such as sialyl Lewis x (sLex) and sialyl Lewis a (sLea), have been shown to function as selectin ligands (2-4).

E-selectin was originally described as a myeloid cell-specific adhesion protein whose expression on cytokine-activated endothelial cells in vivo was thought to direct the entry of neutrophils and monocytes into sites of acute inflammation (5). However, E-selectin has been found on venules in sites of chronic inflammation that are predominantly associated with infiltrates of mononuclear cells (6,7). Recently, human CD4 memory T cells, but not

virgin cells, have been shown to avidly bind E-selectin (8,9). The capacity to bind E-selectin following the acquisition of the memory phenotype is associated with the expression of the HECA-452 epitope (CLA antigen) on T cells (8,10,11). HECA-452 recognizes the "face" of the carbohydrate moiety on lymphocytes that is bound by E-selectin (12). It has been suggested that the interaction between memory T cells and E-selectin may be important in the preferential accumulation of these lymphocytes in certain inflammatory sites (8-10). Picker et al. have extended this hypothesis, based on their observation that E-selectin appears to be preferentially expressed on venules in the skin, and proposed that E-selectin serves as a vascular addressin for lymphocyte recirculation through dermal tissue (8,10). The testing of these hypotheses has not been done due to the fact that all information obtained thus far has been with human cells and not with cells from animal models.

In many animals, including humans, γ/δ T cells are thought to have the capacity to recirculate through epithelial cell-associated tissues (13,14), such as gut and skin, versus a preference of conventional T cells (α/β) for secondary lymphoid organs (15). In particular, γ/δ T cells in ruminants exhibit a considerable capacity to home to the skin (14). Since E-selectin may be a skin-specific addressin, we tested if ruminant γ/δ T cells represent another lymphocyte subset that binds E-selectin. If true, the role of these interactions in vivo could eventually be tested.

We show that bovine γ/δ T cells avidly bind human E-selectin-transfected L-cells. Unlike human CD4 T cells, prior differentiation or acquisition of the HECA-452 epitope is not required for binding. In addition, neuraminidase or protease treatment of the γ/δ T cells blocks adhesion, but known adhesion proteins, such as CD18, CD44, and, importantly, L-selectin, appear to play no role in binding E-selectin. A single 250 kDa glycoprotein, isolated from detergent lysates of γ/δ T cells using an E-selectin affinity column, may serve

as the E-selectin receptor on the lymphocyte. The interaction defined here appears important in vivo, since upregulation of E-selectin expression on bovine dermal vessels by injection of TNF-α increases the accumulation of γ/δ T cells on the same vessels.

These results provide the first demonstration that lymphocytes in an animal model bind E-selectin. Thus, the role of this interaction in vivo can be tested. In addition, the γ/δ T cell's receptor for E-selectin may represent a member of a potentially new family of highly glycosylated surface proteins that serve as high affinity ligands for selectins.

Materials and Methods

Animals

Mixed-breed cattle, ranging in age from one to four weeks, were purchased from local producers and housed at the Montana State University large animal facilities at the Veterinary Molecular Biology Laboratory. Human and bovine peripheral blood was collected into heparinized tubes by venipuncture, and the mononuclear cells (PBMC) were separated by centrifugation through ficoll-hypaque (Histopaque 1077, Sigma, St. Louis, MO). Bovine tissue samples were collected from the animals upon necropsy.

Reagents

The mAbs used in this report have been described elsewhere. DREG-56 is a mouse mAb that recognizes human (16) and bovine (17) L-selectin. IL-A29 (ATCC, Rockville, MD) and BAQ4A (VMRD, Pullman, WA) are mouse mAbs that recognize the Workshop Cluster 1 (WC1) antigens (also referred to as T19) specifically expressed by bovine, ovine, and caprine γ/δ T lymphocytes (18-22). Hermes 3 (23-25) is a mouse mAb that recognizes

CD44 (HCAM) on human leukocytes and crossreacts with bovine lymphocytes (17). 60.3 (generously provided by John Harlan, University of Washington) is a mouse mAb that recognizes CD18 on human leukocytes (26) and crossreacts with ruminant leukocytes. EL-246 (27) and CL2 (28) are mouse mAbs that recognize the SCR and lectin domains of human E-selectin, respectively. EL-246 also crossreacts with bovine L- (27) and E-selectin (Jutila, M.A., personal observation). EL-81 is a mouse mAb that recognizes a nonfunction-blocking epitope on human E-selectin (27). CSLEX is a mouse mAb that recognizes the Lewis carbohydrate derivative sLex (29), and it also crossreacts in the ruminant (Jutila, M.A., personal observation). HECA-452 is a rat mAb that recognizes a carbohydrate moiety expressed on high endothelial cell venules (HEV) (30), myeloid cells (11), and the CLA antigen (12) expressed on a subset of human memory T cells. HECA-452 also crossreacts with lymphoid tissue postcapillary venules in the ruminant (Jutila, M.A., personal observation).

In vitro binding assay

In all binding assays, human E-selectin-transfected L-cells (E-selectin-L-cells) (generously provided by Kei Kishimoto, Boehringer Ingelheim) or the parent cell line control were grown on eight-well Labtek slides (Miles Scientific, Naperville, IL) as previously described (27). Approximately $5x10^5$ PBMC, resuspended in 400 ul HB101 (Irvine Scientific, Santa Ana, CA) serum-free media, were allowed to adhere to the transfectants at room temperature for 30 minutes under constant rotation; the slides were then washed in HBSS (JRH Biosciences, Lenexa, KS) and fixed in 1% glutaraldehyde (Fisher Scientific, Fair Lawn, NJ). All mAbs used in the blocking assays were at a final concentration of 50 ug/ml. Neuraminidase (Sigma, St. Louis, MO) treatment (1 U/ml in 50 mM NaOAc, pH 5.5, 154 mM NaCl, 4 mM $CaCl_2$) of the PBMC was performed at 37°C

for 30 minutes. Similarly, PBMC were treated with chymotrypsin and trypsin (Sigma, St. Louis, MO) (100 U/ml in HBSS) for 60 minutes at 37°C. For the specific removal of leukocyte L-selectin, PBMC were treated with low-dose chymotrypsin (0.5U/ml) or the chemical crosslinker BS^3 (Pierce, Rockford, IL), as previously described (17,31,32). Following the various treatments, the PBMC were washed in HBSS, and the binding assay was performed as described above. All control experiments were performed under the same conditions as the treated cells, but without addition of the enzyme. Following the binding assay, γ/δ T cells were revealed by immunoperoxidase staining using a biotin-labelled IL-A29 mAb, streptavidin peroxidase (Tago, Burlingame, CA), and amino-ethyl carbazol (AEC) (Sigma, St. Louis, MO).

Immunofluorescence staining and fluorescent activated cell sorting (FACS) analysis

The immunofluorescence staining procedure was carried out in 4 ml tubes (Becton Dickinson). Typically, $1x10^6$ cells per tube were initially incubated in 2% rabbit serum for 10 minutes on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 ug/ml (or undiluted culture supernatant) for 20 minutes on ice. After washing, bound antibodies were revealed by incubation with PE conjugated F(ab)'2 goat anti-mouse Ig (Tago, Burlingame, CA). FACS analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA), as previously described (31-34). In all analyses, lymphocytes were identified by their unique forward and side light scatters. For two-color analysis, FITC-conjugated IL-A29 was used after staining with the primary mAb. Before cells were stained with the FITC-conjugated mAb, they were treated with 10% mouse serum to block any available anti-mouse Ig binding sites of the second stage. Data were collected from 10,000 cells and are presented as contour plots.

EL-81-E-selectin Sepharose 4B column preparation

The nonfunction-blocking, anti-E-selectin mAb EL-81 (5mg) was coupled to CNBr-activated Sepharose 4B beads (Pharmacia LKB, Uppsala, Sweden) (1.5 ml swollen bead volume) per manufacturer's instructions. Approximately $3x10^8$ E-selectin-L-cells were lysed in 2% NP-40 (Sigma, St. Louis, MO), 0.1 M NaCl (Sigma, St. Louis, MO), 10 mM HEPES (Mediatech, Washington, DC), 1 mM $CaCl_2$ (Sigma, St. Louis, MO), 1 mM $MgCl_2$ (Sigma, St. Louis, MO), 5 mM NaN_3 (Sigma, St. Louis, MO), with 10 ug each of pepstatin, leupeptin, chymostatin, antipain, benzamidine HCl, 1,10 phenanthroline, and 1 mM PMSF (Sigma, St. Louis, MO), and then centrifuged at 10,000g for 15 minutes. The lysate was incubated on the EL-81 column for 24 hours at 4°C. The EL-81-E-selectin column was then eluted with 0.1% NP-40, 0.1 M NaCl, 10 mM HEPES, 5 mM EDTA (Sigma, St. Louis, MO), 5mM NaN_3 and 1mM PMSF to remove residual binding proteins left from the E-selectin-L-cell lysate, and extensively washed with 0.1% NP-40, 0.1 M NaCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM NaN_3 and 1 mM PMSF.

Purification of the γ/δ T cell E-selectin receptor

Bovine PBMC were collected, adherent cells removed by incubation in a polystyrene flask at 37°C plus 10% CO_2 for 1 hour, and the nonadherent cells ($5x10^8$) lysed as described above for the E-selectin-L-cells. Equivalent numbers of a mouse pre-B tumor cell line (L1.2) or a nonhematopoietic tumor cell line (L-cells) were lysed and used as controls for the E-selectin affinity column procedure. Lysates were precleared over an EL-81 column (no E-selectin) for 24 hours at 4°C. The precleared filtrate was collected, added to the EL-81-E-selectin column, and incubated for 24 hours at 4°C. The EL-81-E-selectin column filtrate was collected, the column rinsed extensively with wash buffer and eluted with 5 mM EDTA elution buffer. Samples were solublized in 6x Laemmli's sample buffer,

electrophoresed through an 8% SDS-polyacrylamide gel under reducing or nonreducing conditions, and detected by silver stain analysis (Bio-Rad, Richmond, CA) per manufacturer's instruction.

Neuraminidase digestion of purified protein

Protein purified from the EL-81-E-selectin column was neuraminidase digested, similar to the conditions described by Moore et al. for optimal sialic acid removal (35). Protein containing EDTA elution fractions were consolidated and the buffer exchanged into a neuraminidase reaction buffer (0.15 M NaCl, 50 mM acetate, pH 6.0, 9 mM $CaCl_2$, 0.005 mM NaN_3, 0.005% NP-40) by use of a Centricon 30 concentrator (Amicon, Beverly, MA). The digestion was performed at 37°C for 10 hours in the presence or absence of 500 mU/ml neuraminidase from *Arthrobacter ureafaciens* (Calbiochem, La Jolla, CA).

TNF-α stimulation of bovine skin

Approximately 5-10 ug of mouse TNF-α (Genentech, San Francisco, CA) in 200 ul sterile HBSS was injected intradermally to raise a diffuse bleb. Tissue from stimulated and unstimulated (contralateral) regions were collected and frozen in O.C.T. medium (Miles Inc., Elkhart, IN). Six um skin sections were cut, fixed in acetone, and blocked with 10-15% rabbit serum for 20 minutes. γ/δ or α/β T cells were revealed by immunoperoxidase staining using the IL-A29 or CC42 mAbs, respectively. E-selectin expression was detected by the EL-246 mAb. The primary mAbs were revealed by second-stage biotinylated anti-mouse antibody (Tago, Burlingame, CA); streptavidin peroxidase; and AEC. Tissue sections were also incubated with isotype-matched negative control mAbs to determine any nonspecific binding of antibodies and/or endogenous peroxidase development of the substrates.

Results

Bovine γ/δ T cells bind E-selectin cDNA-transfected mouse L-cells

Isolated bovine mononuclear cells were tested for their ability to bind mouse L-cells transfected with human E-selectin cDNA (E-selectin-L-cells). The binding assay was performed under rotation at room temperature. Following the assay and fixation of bound cells, the γ/δ T lymphocytes were revealed by immunoperoxidase staining using a lineage-specific, anti-WC1 mAb. As shown in figure 19A, bovine γ/δ T cells avidly bound the E-selectin-L-cells, but not the nontransfected parent cell line control (Figure 19B). Immunoperoxidase analysis demonstrated that γ/δ T cells represented greater than 90% of the mononuclear cells, when isolated from neonatal animals, that bound to the transfectants. Monocytes and, perhaps, memory T cells made up essentially the rest of the binding population. Like their human counterparts (5), bovine neutrophils also bound the E-selectin transfectants (data not shown).

To address what percentage of the γ/δ T cell population has the capacity to bind E-selectin, a mixed PBMC suspension was sequentially passed over different monolayers of the E-selectin transfectants. Greater than 90% of the γ/δ T cells bound E-selectin and were removed from the PBMC population (data not shown).

Characterization of the binding interaction between γ/δ T cells and E-selectin

We initially tested the effects of anti-E-selectin mAbs on the binding of γ/δ T cells to E-selectin. EL-246 and CL2, which recognize the SCR and lectin domains of E-selectin (27,28), respectively, effectively blocked the binding interaction (Table 5). EL-81, which is a nonblocking, anti-E-selectin mAb (27), had little effect (Table 5). Neuraminidase treatment of the γ/δ T cells or addition of 5 mM EDTA to the binding assay also abrogated

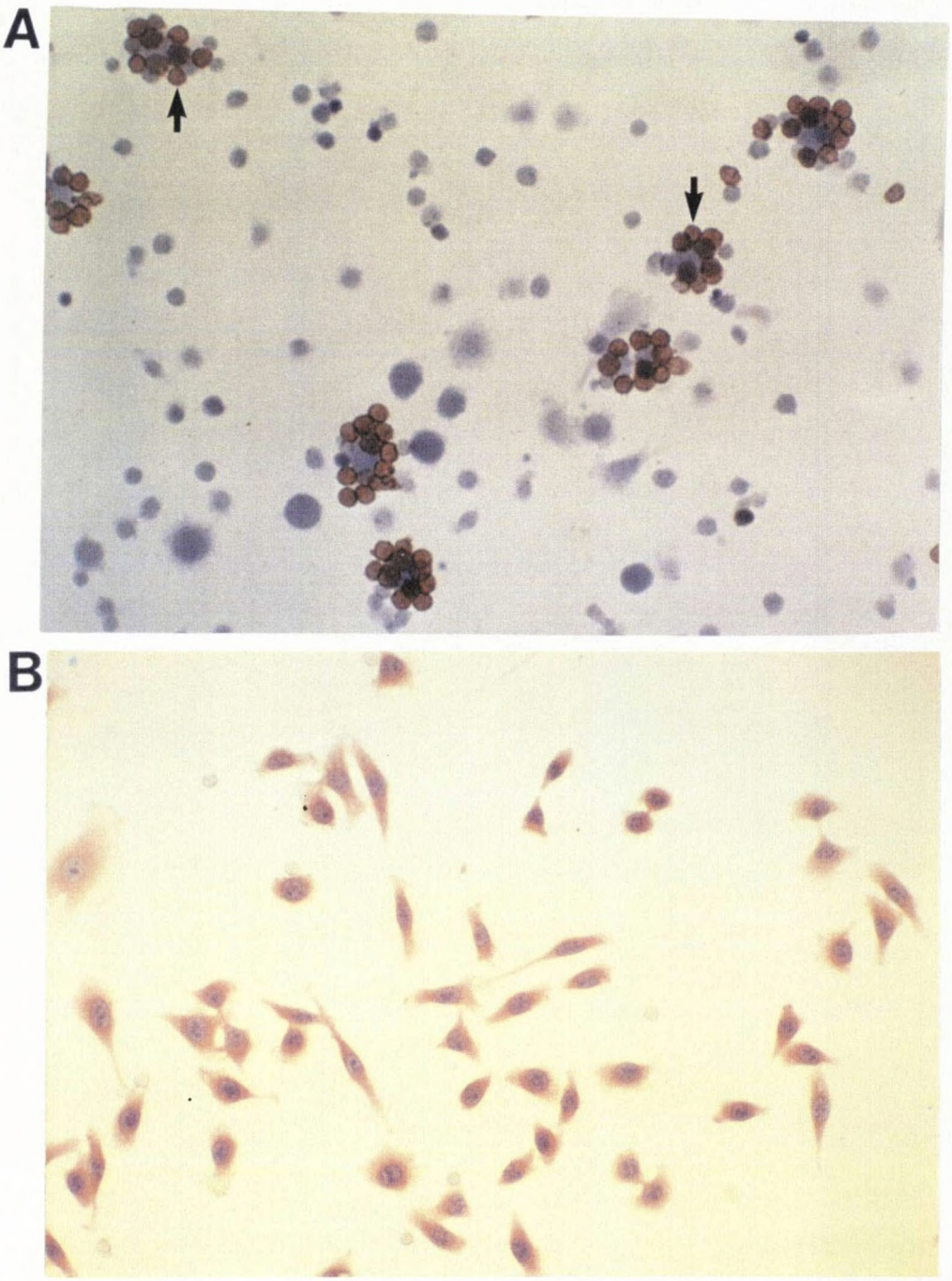


Figure 19. Bovine γ/δ T cells bind E-selectin-transfected L-cells. Bovine PBMC, isolated from neonatal animals, were incubated with the E-selectin-L-cells (A) or the parent cell line control (B) under nonstatic conditions at room temperature as described in Materials and Methods. The γ/δ T cells were revealed by immunoperoxidase staining using the mAb IL-A29 (arrow), which recognizes the lineage-specific WC1 antigen. Approximate magnification = 200x.

adhesion (Table 5). These results demonstrate that E-selectin supports the adhesion of γ/δ T cells and that the binding interaction is dependent upon divalent cations. Furthermore, the apparent affinity necessary for γ/δ T cells to adhere to the E-selectin-L-cells during the rotational binding assay requires sialylated carbohydrates on the lymphocyte's cell surface. These characteristics are similar to other previously defined leukocyte/E-selectin interactions (2,36-40).

Table 5. Characterization of the binding interaction between γ/δ T cells and E-selectin.

	# of γ/δ T cells/L-cell[c]		% inhibition
Treatment of	Treated	Control	of binding[d]
E-selectin-L-cells[a]			
EL-246	0.9+/-0.1	4.5+/-1.0	79+/-3.5
CL2	1.6+/-0.3	4.5+/-1.0	64+/-1.1
EL-81	4.8+/-1.0	4.5+/-1.0	n.d.
EDTA	0	5.1+/-0.5	100
γ/δ T cells[b]			
neuraminidase	0.1+/-0.1	2.8+/-1.6	98+/-0.5

a) E-selectin-transfected L-cells were pretreated with the anti-E-selectin mAbs EL-246, CL2, or EL-81 at 50ug/ml for 30 minutes, and the binding assay was performed as described in Materials and Methods. Five mM EDTA was added to the E-selectin-L-cells prior to the addition of the PBMC. γ/δ T cells, bound to the E-selectin-L-cells following the binding assay, were revealed by immunoperoxidase staining with the lineage-specific mAb biotin-conjugated IL-A29.
b) PBMC were treated with neuraminidase (1U/ml) prior to their addition to the E-selectin-L-cells.
c) γ/δ T cell binding to the E-selectin-L-cells was quantified by counting three separate fields at 20x power. The mean number of γ/δ T cells per E-selectin-L-cell was determined for control and treated cells. The values given represent the mean from three separate

experiments +/- SE.
d) Percent inhibition of binding = (control mean-treated mean/control mean) x 100. The values given represent the mean inhibition from three separate experiments +/- SE. If no significant inhibition of binding occurred then n.d. is indicated for none detected.

Next we tested if bovine γ/δ T cells are recognized by mAbs that bind carbohydrates which support E-selectin adhesion in other systems, such as CSLEX and HECA-452 (8,10,36-40). In other studies we have found that HECA-452 crossreacts in the ruminant and stains peripheral lymph node postcapillary venules (Jutila, M.A., unpublished observations), which is similar to its reactivity in the human (30). However, we found that HECA-452 did not recognize bovine PBMC (Figure 20a). The CSLEX mAb also crossreacted in the cow, but stained less than 5% of bovine γ/δ T cells (Figure 20b). The lack of staining of bovine γ/δ T cells was not due to poor antibody preparations, since the HECA-452 and CSLEX mAbs did recognize human PBMC (Figure 20c and d, respectively). These results suggest that the sialylated carbohydrate structures recognized by E-selectin on γ/δ T cells are antigenically different than previously characterized E-selectin carbohydrate ligands.

In other systems, glycolipids and glycoproteins have been shown to present E-selectin carbohydrate ligands (41,42). To address whether protein or lipid is important to the γ/δ T cell presentation of E-selectin ligands, the effects of different proteases on the binding interaction were tested. Table 6 shows that chymotrypsin or trypsin treatment of bovine PBMC blocked binding by an average of 72% and 35%, respectively. Due to the protease sensitivity of the binding interaction, we examined the effects of three function-blocking antibodies against known glycoprotein adhesion molecules on γ/δ T cells (L-selectin, CD18, and CD44) for an inhibitory effect on E-selectin binding. Table 6 shows that none of these antibodies effectively blocked binding. Because L-selectin on neutrophils has been

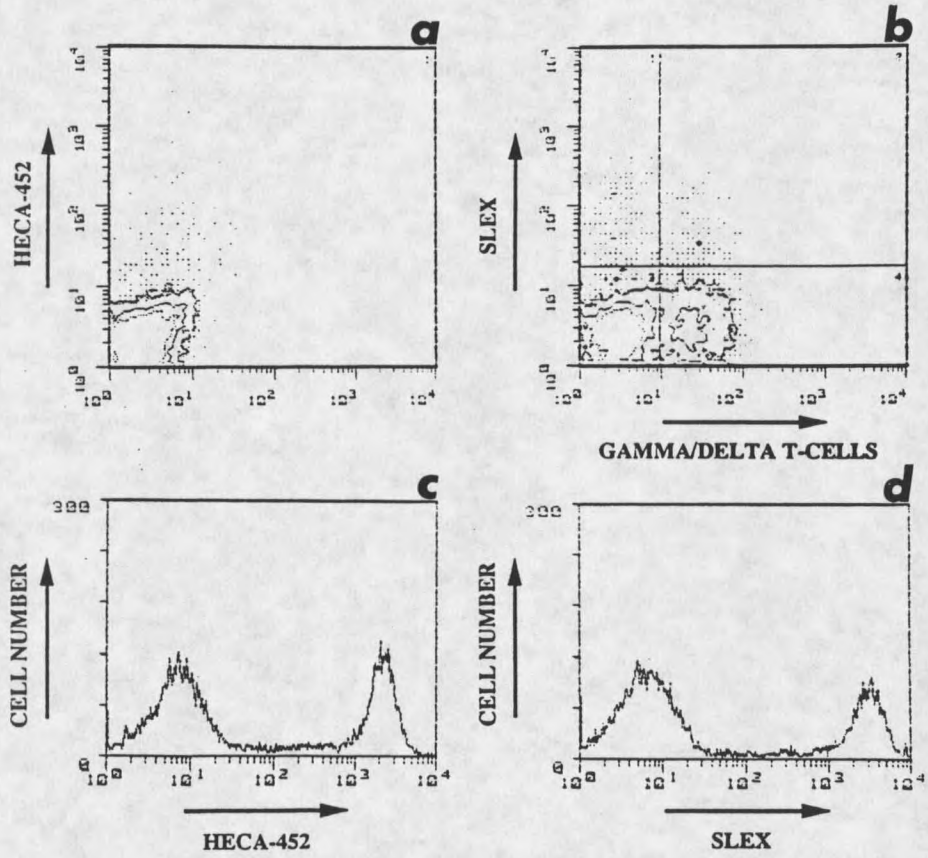


Figure 20. Bovine PBMC lack significant expression of the sLex and HECA-452 carbohydrate moieties. PBMC were separated and stained for FACS analysis by the mAb HECA-452. For two-color FACS analysis, the γ/δ T cells were stained by the mAbs FITC-conjugated IL-A29 and CSLEX as described in Materials and Methods. Panel A shows the level of HECA-452 expression on PBMC, which is equivalent to the level of staining demonstrated by an isotype-matched negative control antibody. Panel B shows the level of sLex expression on γ/δ and non-γ/δ T cells. Less than 5% of the γ/δ T cells expressed sLex after background fluorescence with an isotype control mAb was subtracted out of the percentage of positive staining cells. Panels C and D show sLex and HECA-452 expression on human PBMC, respectively.

proposed to be a ligand for E-selectin in the human, (28,42) and since γ/δ T cells express 2-5x the level of this receptor as other leukocytes (see Chapter 2), we examined the effects of other treatments that block L-selectin function. BS^3 or low-dose chymotrypsin (0.5U/ml) treatments reduced the expression of L-selectin on the γ/δ T cells by >95%

Table 6. The effects of function-blocking mAbs against γ/δ T cell adhesion glycoproteins on E-selectin binding.

Treatment of γ/δ T cells	Adhesion protein	# of γ/δ T cells/L-cell[d] Treated	Control	% inhibition of binding[e]
Hermes 3[a]	CD44	3.4+/-0.6	3.5+/-0.2	n.d.
60.3	CD18	3.5+/-0.5	3.5+/-0.2	n.d.
DREG-56	L-selectin	4.3+/-0.3	3.5+/-0.2	n.d.
chymotrypsin (0.5U/ml)[b]	L-selectin	1.9+/-0.5	2.2+/-0.3	n.d.
BS^3	L-selectin	2.0+/-0.6	2.2+/-0.3	n.d.
chymotrypsin (100U/ml)[c]		1.0+/-0.4	4.6+/-0.7	72+/-9.3
trypsin (100U/ml)		3.2+/-1.1	4.6+/-0.7	35+/-11

a) γ/δ T cells were pretreated with the mAbs Hermes-3, 60.3, or DREG-56 at 50ug/ml for 30 minutes, and the binding assay was performed as described in table 5.
b) γ/δ T cell L-selectin was removed from the cell surface by treatment of these cells with low-dose chymotrypsin or the chemical crosslinker BS^3 as described in Materials and Methods.
c) γ/δ T cell surface membrane proteins were removed by protease treatment with chymotrypsin or trypsin as described in Materials and Methods.
d) γ/δ T cell binding to the E-selectin-L-cells was quantified as described in table 5.
e) Percent inhibition of binding = (control mean-treated mean/control mean) x 100. The values given represent the mean inhibition from three separate experiments +/- SE. If no significant inhibition of binding occurred then n.d. is indicated for none detected.

(data not shown), which is the same result as seen with other leukocytes (17,31,32,42); but these agents had no significant effect on the binding interaction (Table 6). Our results

demonstrate that both sialylated carbohydrates and a protein component are important for γ/δ T cell binding to E-selectin, but that certain known adhesion molecules are likely not involved (further evidence provided below).

E-selectin binds a single glycoprotein in detergent lysates of γ/δ T cells

Purified E-selectin was immobilized on a supporting matrix to isolate putative receptors from a detergent lysate of γ/δ T cells. Sepharose 4B beads coupled to a nonfunction-blocking anti-E-selectin antibody (EL-81) were used to capture detergent-solublized E-selectin derived from the E-selectin-L-cells. γ/δ T cells were lysed in NP-40 to isolate functional cell surface membrane complexes, precleared on a EL-81 column, which removed a considerable amount of protein, and then incubated on the EL-81-E-selectin column. Material bound to both the preclearing and E-selectin columns was eluted using a 5 mM EDTA elution buffer. As shown in figure 21A, a predominant molecule with an approximate molecular weight of 250 kDa was extracted by the EL-81-E-selectin column and detected in the elution fractions 3-6 (lanes 9-12) by nonreducing SDS-PAGE and silver stain analysis. The 250 kDa band was faintly evident in the filtrate from the preclearing column, but undetected in the EDTA elution fractions from the same column. The 250 kDa band was not detected in the E-selectin column filtrate (Figure 21A); however, all other bands seen in the filtrate off the preclearing column were still present (compare lanes 2 and 6). Some minor protein bands were present in the EL-81-E-selectin elution fractions as well. For instance, a molecule with a molecular weight of 69 kDa was detected; however, this molecule was also evident in the elution fractions off the preclearing column and the filtrate from the E-selectin column (Figure 21A). Thus, we believe it interacted with EL-81 and/or the supporting beads, but not E-selectin. A continuous background band was also seen at approximately 46 kDa, which was generated by a contaminating protein within the

Laemmli's sample buffer. The E-selectin affinity-purified molecule migrated at approximately 280 kDa under reducing conditions (Figure 21B), which consisted of solublization at 37°C with 2-mercaptoethanol due to the 250 kDa molecules sensitivity to boiling (it may aggregate).

As established above, the binding interaction between γ/δ T cells and E-selectin is dependent upon sialic acid. Related studies by Moore et al. (35) describing a myeloid glycoprotein receptor for P-selectin have demonstrated the importance of sialic acid to the

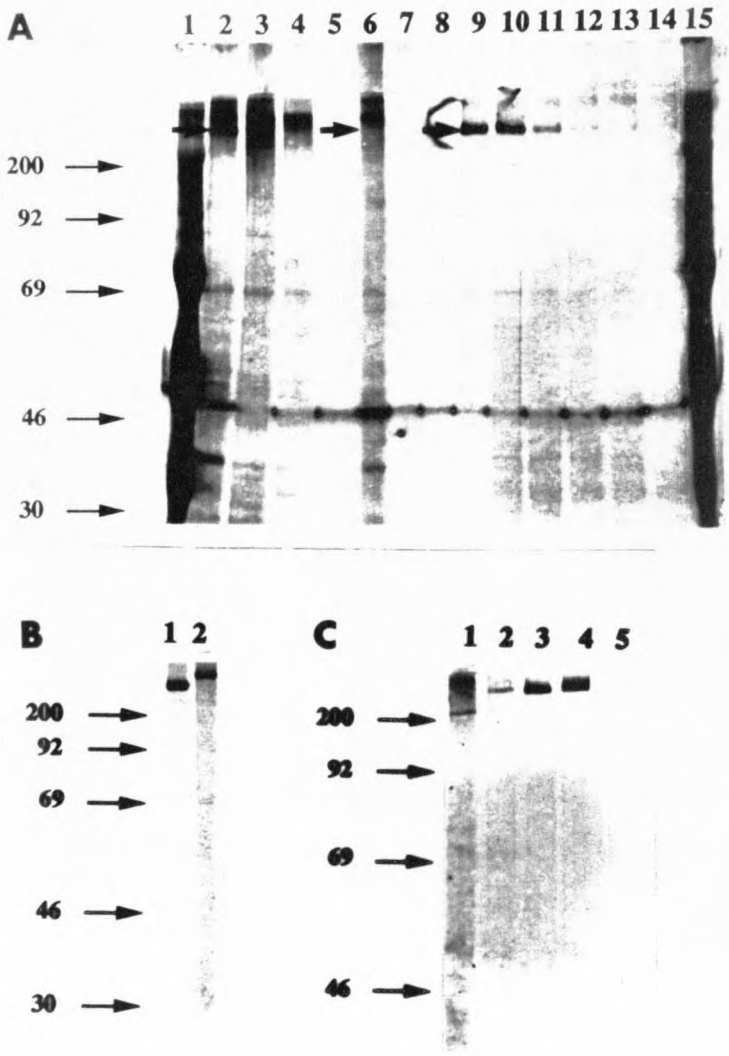


Figure 21. Silver stain analysis of a single γ/δ T cell glycoprotein isolated from an E-selectin affinity column. (A) Samples from the respective steps involved in the purification of the E-selectin ligand from a γ/δ T cell lysate were electrophoresed on 8% SDS-polyacrylamide gels under nonreducing conditions and revealed by silver stain analysis as described in Materials and Methods. Lanes 2 and 3 represent the filtrates from a γ/δ T cell lysate sequentially passed over the EL-81 preclearing column twice [Lane 2 = first passage, Lane 3 = second passage (arrow marks the presence of the 250 kDa band)]. Lane 4 represents a sample of the consolidated EDTA elution fractions 3, 4, and 5 from the EL-81 preclearing column. Lane 5 represents 6x Laemmli's sample buffer plus running buffer. Lane 6 represents the filtrate of the precleared γ/δ T cell lysate collected from the EL-81-E-selectin column (arrow marks the absence of the 250 kDa band). Lanes 7-14 represent the EDTA elution fractions 1-8, respectively, from the EL-81-E-selectin column (arrow marks the presence of the 250 kDa band). Lanes 1 and 15 represent molecular weight markers, which are designated on the left of the figure by their respective mass. (B) The 250 kDa product was run on an 8% polyacrylamide gel under nonreducing (lane 1) and reducing (lane 2) conditions. (C) Equal concentrations of the 250 kDa product were neuraminidase-digested or sham-treated then re-isolated by the E-selectin affinity column; lanes 5 and 4, respectively. An equivalent number of a mouse pre-B (L1.2 cells) or non-hematopoietic (L-cells) tumor cell line to γ/δ T cells were NP-40 solublized and affinity column extracted as described above. EDTA elution fractions from the EL-81-E-selectin column from each lysate were consolidated and equal sample volumes analyzed; lane 1 = L-cells, lane 2 = L1.2 cells, lane 3 = γ/δ T cells.

function of their molecule as well. Using the same neuraminidase enzyme, buffer, and incubation conditions determined by Moore et al. to be optimal for the removal of sialic acid residues from their glycoprotein, we digested the purified 250 kDa molecule to determine if it could be re-isolated by the E-selectin affinity column. As shown in figure 21C, reactivity of the 250 kDa molecule with E-selectin was greatly diminished by prior neuraminidase digestion, though treatment of an equal concentration of the molecule under the same conditions without neuraminidase had no apparent effect on the binding interaction. Neuraminidase digestion of the 250 kDa molecule decreased its mobility during SDS-PAGE (equivalent to 300 kDa, data not shown), which is characteristic of heavily sialylated glycoproteins (43,44) and similar to what has been described for the glycoprotein receptor for P-selectin (35). However, only the 250 kDa molecule, and not the digested product (300 kDa), was able to interact with E-selectin. These results show that there are only a

limited number of protein species (even under these low stringency conditions) that can be bound by E-selectin. Furthermore, the 250 kDa molecule's specificity for E-selectin is EDTA and neuraminidase sensitive, the same as γ/δ T cell binding to the E-selectin-L-cells.

We then tested for a similar E-selectin binding molecule in lysates generated from two tumor cell lines (L1.2 and L cells). L1.2 cells (mouse pre-B tumor cell line) demonstrated a low avidity capacity to interact with the E-selectin-L-cells (data not shown). Five x10^8 L1.2 cells were lysed and run over the preclearing and E-selectin columns as described above for the γ/δ T cells. The EDTA elution fractions off the EL-81-E-selectin column were consolidated, and the same was done with elution fractions from a γ/δ T cell lysate of equivalent cell numbers. An equal sample volume (reflecting material from the same number of cells) from each of the two preparations was examined by silver stain analysis. Interestingly, a 250 kDa molecule was isolated from the L1.2 cells; however, the expression level was far less than on γ/δ T cells (Figure 21c), which correlated with the L1.2 cells' low E-selectin binding capacity (approximately 1/20 the capacity of γ/δ T cells). Similar analysis was done with L cells, a mouse nonhematopoietic tumor cell line, from which the 250 kDa molecule was not detected (Figure 21c). The 300 and 200 kDa proteins detected in the L-cell elution fractions (Figure 21c) were eluted off the preclearing column as well, indicating it was interacting with EL-81 and/or the supporting matrix, but not E-selectin (data not shown).

Up-regulation of E-selectin in bovine skin is associated with increased γ/δ T cell localization

Animals of approximately eight weeks of age were stimulated by an intradermal injection of 5-10 ug TNF-α, a known inducer of E-selectin expression (5,45), to examine γ/δ T cell recruitment. Sections cut from control and stimulated skin samples were stained by immunoperoxidase using the γ/δ T cell-specific mAb IL-A29, the α/β T cell-specific

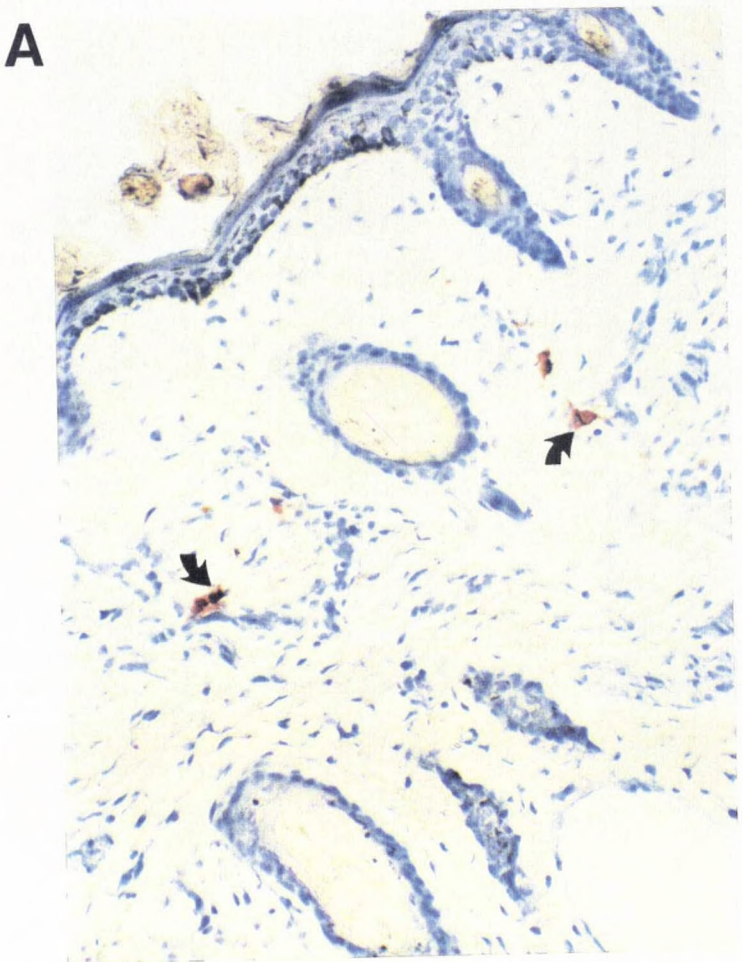
mAb CC42, or the anti-E-selectin mAb EL-246. Figure 22a shows γ/δ T cells from a representative region of control skin. Figure 22b shows a representative region of TNF-α stimulated skin stained with the anti-E-selectin mAb EL-246, note the intense luminal expression of E-selectin on the dermal blood vessel. A serial section of this same vessel shows that γ/δ T cells avidly adhered to the E-selectin positive endothelial cells (Figure 22c). Using serial sections, we compared the ratio of γ/δ T cells to α/β T cells actively bound to the luminal surface of venules in TNF-α inflamed skin (Figure 22d and e). This analysis allowed for the quantitation of newly recruited lymphocytes versus cells (both γ/δ and α/β T cells) that were already in the tissue prior to induction of inflammation. The γ/δ T cell to α/β T cell ratio in peripheral blood was approximately 0.7:1, but the ratio found in inflamed dermal venules was 3.7:1 (determined from analysis of >200 cells). These results suggest that there is a correlation between the upregulation of E-selectin expression and increased γ/δ T cell accumulation.

Discussion

E-selectin was originally defined as an inducible endothelial cell adhesion protein for myeloid cells (5,45) and is believed to be important in regulating acute inflammatory responses. Recent studies have expanded the role of E-selectin to serving as a putative vascular addressin in certain chronic inflammatory processes for a subset of human memory T cells (8,9,10,11). We add to these studies by demonstrating the capacity of ruminant γ/δ T cells from neonatal and mature animals to bind E-selectin. This interaction is supported by our in vivo studies in which induction of E-selectin expression by TNF-α (as well as LPS and the *Mycobacterium* derived protein PPD, data not shown) in bovine skin is associated with an influx of γ/δ T cells at the same site. Our analyses are directly relevant to the human, since a significant percentage of human γ/δ T cells express the HECA 452 epitope which Picker et al. and Berg et al. (2,8,10-12 and see below) have shown

correlates precisely with the ability of lymphocytes to bind E-selectin, and we have found that a small percentage of human lymphocytes which bind E-selectin are stained by anti-γ/δ TCR mAbs (data not shown). Thus, E-selectin has the potential of regulating many diverse leukocyte-endothelial cell interactions that occur in different inflammatory settings.

Norton et al. have suggested that particular tissues may constitutively express low levels of E-selectin on certain venules (46,47). If true, E-selectin may be involved in leukocyte extravasation in the absence of overt inflammation. This hypothesis is directly



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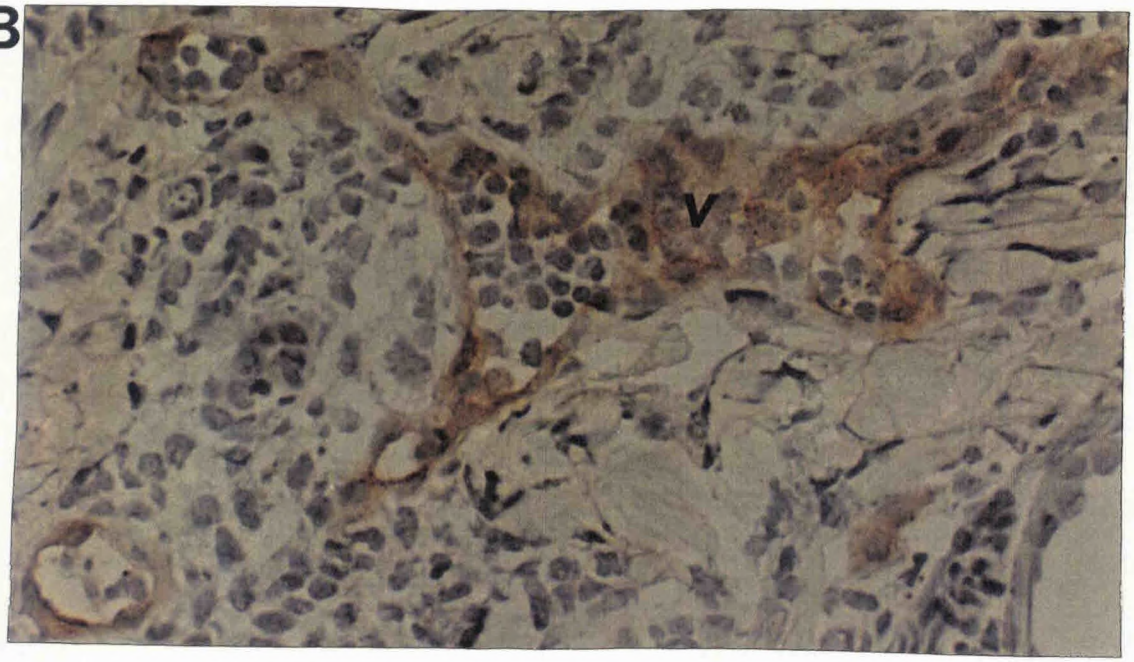

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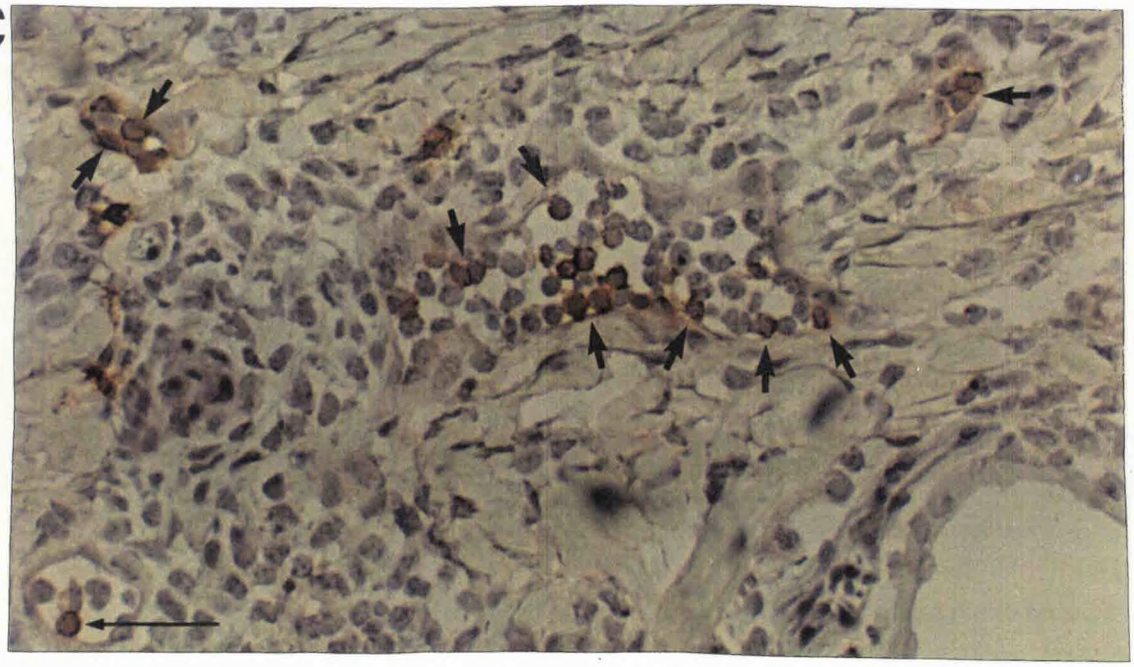

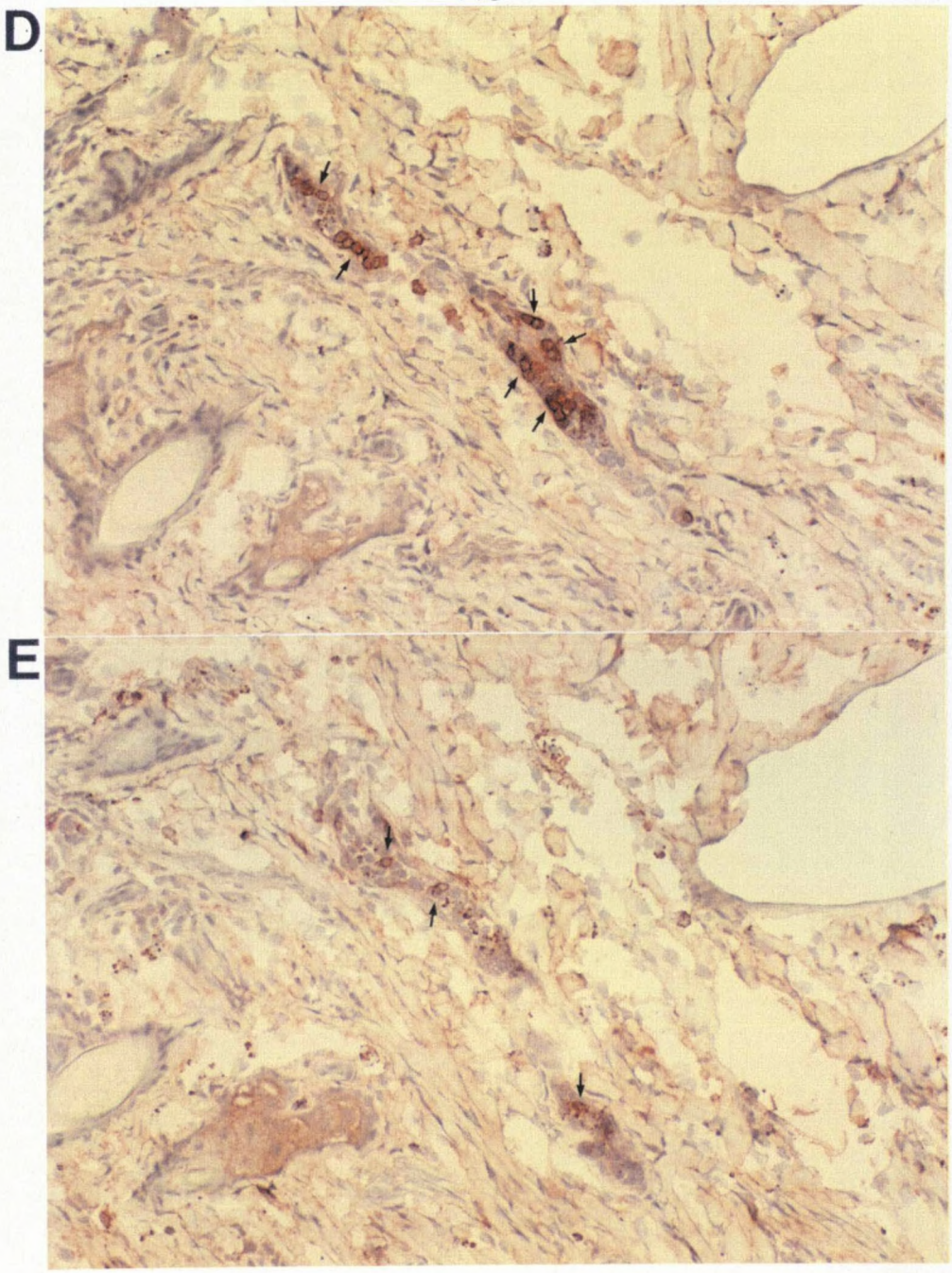

Figure 22. TNF-α -induced, endothelial cell E-selectin expression in skin is associated with an accumulation of γ/δ T cells at the same site. Bovine skin was stimulated for 6 hours by intradermal injection of 10 ug TNF-α. (A) Control skin stained for γ/δ T cells (arrow). (B and C) Serial sections of stimulated skin stained for E-selectin expression on the dermal blood vessels (v) and γ/δ T cells (arrow), respectively. (D and E) Serial sections of stimulated skin stained for γ/δ and α/β T cells (arrow), respectively, accumulated in open blood vessels. Skin samples were stained by immunoperoxidase using the γ/δ T cell-specific mAb IL-A29, the α/β T cell-specific mAb CC42, or the anti-E-selectin mAb EL-246.

relevant to γ/δ T cells. The tissues that have been shown to constitutively express low levels of E-selectin are the skin and gut: both are sites of γ/δ T cell localization (13,14). Preliminary analysis suggests that γ/δ T cells bind E-selectin more avidly than other leukocytes (data not shown). If true, low levels of E-selectin on certain venules may preferentially recruit γ/δ T cells and account for their localization in uninflamed skin and gut. Indeed, E-selectin may serve the same role in certain extralymphoid tissues as the vascular addressins play in lymph nodes and Peyer's patches.

In this study, we provide a preliminary characterization of the adhesive mechanism used by γ/δ T cells to bind E-selectin. A previously described component of leukocyte receptors for E-selectin is sialylated carbohydrates. Sialyl Lewis x (sLex) on neutrophils (36,39) and the HECA-452 epitope (CLA antigen) on human memory lymphocytes (8,10) are examples of these carbohydrate structures. Sialylated carbohydrates appear important in the binding of γ/δ T cells to E-selectin as well, since neuraminidase treatment of the lymphocytes inhibits the interaction. However the CSLEX and HECA-452 mAbs do not recognize γ/δ T cells, suggesting that additional biological carbohydrate structures, antigenically different from what has been described on myeloid cells and CLA^+ lymphocytes, are recognized by E-selectin.

The carbohydrate ligands are only a partial representation of the entire receptor for selectins. It is likely that the biological receptors for the selectins would also be composed of lipid or protein. Importantly, both P- and L-selectin have been shown to bind specific cell surface glycoproteins with high affinity (35,48). The complete significance of this protein component has not been determined. Protein may provide for a "scaffolding" in which carbohydrate structures are presented to the selectins (48). However, it is possible that certain regions of the protein backbone may participate in a protein-protein interaction with the selectins, in addition to the carbohydrate-lectin interaction.

Studies addressing the nature of the biological receptor on leukocytes for adhesion to E-selectin vary considerably in their findings. SLex and the HECA-452 antigen are found on many different glycoproteins and glycolipids on the leukocyte surface, suggesting that a large number of molecules could potentially serve as receptors (42). However, this does not appear to be the case. Activation of neutrophils actually increases sLex expression, but binding to E-selectin is greatly diminished (28,42,49). Thus, there appears to be a restriction in the types of molecules on the leukocyte surface that can bind E-selectin.

Putative glycoprotein receptors for E-selectin have been described on neutrophils. For example, L-selectin may preferentially present sLex on neutrophils to E-selectin. Anti-L-selectin mAbs block neutrophil adhesion to E-selectin cDNA transfectants to the same extent as anti-E-selectin mAbs, suggesting that E- and L-selectin can serve as receptor-ligand pairs (28). Picker et al. extended these observations and found that L-selectin is decorated by sLex, and its preferential localization at the tips of the microvilli and ruffles of the neutrophil cell surface membrane may account for its predominant role in presenting carbohydrates to E-selectin (42). A recent study by Vestweber et al. (50) has described an additional glycoprotein (160 kDa, reducing conditions) that can be isolated from detergent lysates of mouse neutrophils by an E-selectin affinity column. However, an analogous role for L-selectin or any other single glycoprotein in mediating lymphocyte adhesion to E-selectin has not been shown.

We show that protein is a major component of the γ/δ T cell receptor for E-selectin. Treatment of γ/δ T cells with chymotrypsin or trypsin (to a lesser degree) blocks the binding interaction. L-selectin, CD44, and CD18, on γ/δ T cells do not appear to represent the surface receptor. In particular, three different approaches that block L-selectin function had no significant effect on γ/δ T cell/E-selectin adhesion.

To address whether single or multiple cell surface glycoproteins can potentially serve as E-selectin ligands, we examined NP-40 solublized membrane molecules from γ/δ T cells

by an E-selectin affinity column. The affinity isolation approach used here purified a single 250 kDa glycoprotein which required sialic acid and divalent cations for an interaction with E-selectin. A similar size molecule was not found in a lysate of a nonhematopoeitic tumor cell line (L cells). Lysates of a mouse lymphoid tumor cell line (L1.2) did contain the 250 kDa molecule; however, the apparent expression level was much less than that from an equal number of γ/δ T cells. The low expression of the putative E-selectin receptor by the L1.2 cells was not surprising for two reasons: 1) Low numbers of L1.2 cells are capable of binding E-selectin-L-cells under rotation, and 2) a recent study by Vestweber et al. (50) using an E-selectin-IgG fusion protein has identified a predominant 160 kDa molecule and a minor 250 kDa molecule from detergent lysates of mouse leukocytes isolated from bone marrow. The 160 kDa molecule appears to be myeloid specific, but the 250 kDa molecule may have been derived from the minor bone marrow lymphocyte population. In support of this we have also isolated the 250 kDa molecule from human lymphocytes, but not myeloid cells (data not shown). In consideration of the proposed glycoprotein receptors for L- and P-selectin, the γ/δ T cell's receptor for E-selectin may represent an additional member of a potentially new family of highly glycosylated surface proteins that serve as high affinity ligands for selectins.

It was beyond the intent of this study to provide a detailed biochemical characterization of the 250 kDa molecule. Such analysis will include the generation of antibodies to determine the biological significance of this molecule. Antibodies will be necessary to definitively establish a functional role for the 250 kDa molecule at the level of the in vitro binding assay and eventually in vivo. Interestingly, the molecule defined here is related in several ways to two other recently described molecules: 1) The proposed receptor for P-selectin isolated from the surface of neutrophils, which is approximately 250 kDa (under nonreducing conditions) (35); and 2) the WC1 antigens (also referred to as T19)

(14,19,51,52), a recently cloned, lineage-specific, γ/δ T cell surface glycoprotein whose function is unknown (20). Because of the WC1 antigens' selective expression, it has been proposed to be an epithelial-associated tissue homing receptor for γ/δ T cells (22). Immunoprecipitation of the WC1 antigen reveals two predominant molecular species of 165 and 275 kDa (nonreducing conditions), as well as other minor species (19,22). It is possible the 250 kDa glycoprotein defined here represents one of the WC1 isoforms. Our testing of this hypothesis to date has been incomplete; however, anti-T19 mAbs do not block γ/δ T cell adhesion to E-selectin. To confirm the similarity of these three proteins, antibodies generated against our 250 kDa molecule will be tested for antigenic crossreactivity towards the receptor for P-selectin and the WC1 antigens.

In conclusion, our results provide the first demonstration that lymphocytes in an animal model bind E-selectin. Ruminant γ/δ T cells adhere to E-selectin with high avidity and may use a previously undescribed 250 kDa glycoprotein as their receptor. To date, there has been no in vivo evidence that blocking E-selectin alters lymphocyte extravasation. This is because virtually all studies of the interaction between lymphocytes and E-selectin have been done in the human where reagents and assays are readily available, but homing experiments cannot be done. Defining lymphocyte-E-selectin interactions in ruminants provides a significant advancement because of the potential for in vivo analysis. Defining the γ/δ T cell system in ruminants is particularly advantageous because these cells comprise 40-60% of the circulating T cell pool (14), and it can be expected that if their circulatory pathways are blocked a biological effect can be readily measured.

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CHAPTER 5

CONCLUSIONS

1. The in vivo short-term homing assay provides direct evidence that bovine peripheral blood α/β T cells accumulate in secondary lymphoid organs, whereas peripheral blood γ/δ T cells do not, but traffic to epithelial-associated tissues instead. Previously, γ/δ T cell trafficking pathways have only been studied by indirect methods (refer to Chapter 3).

2. Bovine lymphocyte trafficking to PLNs is directed by a primary adhesion event between lymphocyte L-selectin and the PCV MECA-79 Ag. Bovine lymphocyte L-selectin was shown to be functionally, biochemically, and molecularly similar to the human and mouse homologues. In addition, MECA-79 reactivity, under normal conditions, was specific for bovine PLNs. Differences unique to ruminant L-selectin were apparent as well. In both cattle and sheep, the predicted amino acid sequence contained an additional site for a N-linked carbohydrate attachment, though the significance this may convey to the function or regulation of L-selectin has not been addressed. Furthermore, conventional lymphoid trafficking lymphocytes, such as α/β T cells, demonstrated an exquisite separation of lymphocyte homing phenotypes in the different secondary lymphoid compartments. For example, on average 80% of the lymphocytes in PLNs were L-selectin positive compared to 20% in the Peyer's patch, which is a greater restriction of L-selectin expression than what has been described in the human and mouse.

3. Bovine γ/δ T cells express high levels of L-selectin. In newborn animals, peripheral blood γ/δ T cells expressed L-selectin at levels 2-5x greater than peripheral blood α/β T cells. In mature animals, variable numbers of α/β T cells were L-selectin positive, indicative of their different homing phenotypes, whereas γ/δ T cells remained L-selectin positive, though their expression levels decreased.

4. γ/δ T cell L-selectin is functional. Using two separate assays that measure L-selectin function, bovine γ/δ T cells were shown to bind PLN PCV: In one, the Stamper and Woodruff ex vivo binding assay that use frozen sections of mouse PLN, which γ/δ T cells demonstrated appreciable binding to the HEV; and in the other, a two-color immunoperoxidase stain of bovine PLNs, γ/δ T cells could be found lining the vascular wall of MECA-79$^+$ vessels.

5. γ/δ and α/β T cells demonstrate differential short-term regulation of L-selectin. Kinetic analysis revealed that at all time points after PMA activation (1-30 minutes) L-selectin expression remained significantly higher on bovine γ/δ T cells and was downregulated at a slower rate compared with α/β T cells. The inability of γ/δ T cells to efficiently downregulate L-selectin within the rapid time period of lymphocyte interaction with the PCV may contribute to their deficient extravasation into the PLNs. Further studies need to be performed in order to determine which component(s) of the γ/δ T cell L-selectin regulatory pathway is different from that which occurs in α/β T cells; for instance, is it L-selectin itself or the activation/signalling events that direct its downregulation. It appears that L-selectin is identical on γ/δ and α/β T cells. γ/δ T cell L-selectin exhibited the same sensitivity to proteolysis and chemical-crosslinking and mediated the same binding interaction as L-selectin on other lymphocytes.

6. γ/δ T cell secondary adhesion events appear to function properly. CD18 and CD44 were found to be expressed at equivalent levels on both γ/δ and α/β T cells. In addition, the γ/δ T cell secondary adhesion proteins are functional since these cells extravasate from the blood at epithelial locations.

7. γ/δ T cells bind the skin vascular addressin E-selectin. Bovine peripheral blood γ/δ T cells adhered to human E-selectin-expressing L-cells under nonstatic conditions in vitro. This binding interaction was inhibited by anti-E-selectin mAbs. In addition, E-selectin expression on dermal PCV in vivo correlates with increased γ/δ T cell accumulation. Following TNF-α (as well as LPS or PPD) stimulation of bovine skin, γ/δ T cells adhered to the vascular wall of E-selectin-positive vessels with high avidity.

8. Cell-surface carbohydrate on γ/δ T cells as well as the presence of divalent cations are necessary for E-selectin adhesion. Consistent with previous studies using other leukocytes, neuraminidase-treated γ/δ T cells or the addition of EDTA to the binding assay inhibited these cells from binding E-selectin. Since neuraminidase removes sialic acid, the E-selectin ligand on γ/δ T cells may bear carbohydrate structures from the sialylated-Lewis (sLe) family (see Chapter 4). However, several mAbs that recognize these carbohydrates on human leukocytes (and block E-selectin binding) and crossreacted in the cow did not stain bovine γ/δ T cells. Thus, the E-selectin ligand on γ/δ T cells is antigenically different from ligands previously described on other leukocytes.

9. Cell-surface protein on γ/δ T cells is necessary for E-selectin adhesion. Chymotrypsin or trypsin treatment of γ/δ T cells inhibited their binding to E-selectin. However, function-blocking mAbs to known lymphocyte adhesion glycoproteins, such as CD44 and CD18 (β_2

integrins), did not inhibit γ/δ T cell adhesion to E-selectin. Furthermore, L-selectin-specific mAbs or the specific removal of this adhesion protein from the cell surface did not affect γ/δ T cell binding to E-selectin.

10. An E-selectin affinity column purifies a single glycoprotein of 250 kDa from NP-40-solublized, γ/δ T cell membrane proteins. Like γ/δ T cells, the ability of the 250 kDa molecule to bind E-selectin was sensitive to the presence of EDTA or prior neuraminidase digestion. In addition, this putative E-selectin receptor appears to be heavily glycosylated, due to decreased SDS-PAGE mobility following neuraminidase digestion.

In summary, though α/β and γ/δ T cells express functional L-selectin, the γ/δ T cell's recirculation pathway is not directed through PLNs. L-selectin on γ/δ T cells is expressed at higher levels and regulated differently following short-term PMA activation compared to α/β T cells. γ/δ T cells recirculate through epithelial-associated tissues and appear to use a novel 250 kDa glycoprotein receptor to adhere to the vascular addressin E-selectin.

Ongoing studies provide further evidence to support and expand on the above conclusions. Preliminary findings demonstrate that other vascular ligands in addition to E-selectin are recognized by γ/δ T cells and may be involved in γ/δ T cell trafficking to epithelial locations as well. Furthermore, the roles of these primary adhesion events are being examined during certain infectious diseases. Below is an overview of some of these studies.

In Chapter 4, I provided evidence that γ/δ T cells bind E-selectin in vitro and in vivo in the skin. However, to establish more definitively whether this interaction occurs in vivo, it must be blocked by an agent specific to E-selectin or its ligand on the γ/δ T cell. Therefore,

we iv administered a function-blocking anti-E-selectin mAb (EL-246) into newborn calves, as well as isotype control mAbs into other animals, and subsequently induced E-selectin expression in the skin by intradermal injections of several stimulating agents, including TNF-α. In support of our earlier findings, preliminary examination of the injected regions (identified by trapped fluorescent microspheres co-injected with the stimulating agent) from EL-246 administered animals revealed numerous E-selectin-positive vessels (detected by immunoperoxidase staining using second-stage anti-mouse antibody alone due to the luminally bound EL-246); but a dramatically reduced number of γ/δ T cells bound to their vascular wall compared to inflamed sites in control animals. In addition, the number of myeloid cells (neutrophils and monocytes), which previously have been shown to bind E-selectin, were significantly reduced at sites of inflammation in EL-246-iv injected animals compared to controls. Once function-blocking mAbs are developed to the γ/δ T cell E-selectin receptor, they will be tested in vivo as well.

In addition to E-selectin, P-selectin expression can be induced on endothelial cells (and platelets) by certain stimulating agents, such as thrombin. P-selectin was originally described as an inducible vascular ligand for myeloid cells, but recently has been shown to be recognized by certain human T cell subsets (see Chapter 1). Our preliminary studies suggest that bovine γ/δ T cells may recognize P-selectin in addition to E-selectin at sites of inflammation in the skin. Initially, γ/δ T cells were shown to bind P-selectin by an in vitro adhesion assay using thrombin-activated human and bovine platelets as a source of P-selectin. This binding event can be inhibited by pretreatment of the platelets with an anti-human P-selectin mAb. Further evidence that γ/δ T cells bind P-selectin was provided by a P-selectin fusion protein (a combination of human P-selectin and the CH1 and CH2 domains of human immunoglobulin that is used like a mAb), which specifically bound γ/δ but not α/β T cells as determined by FACS analysis (Figure 23).

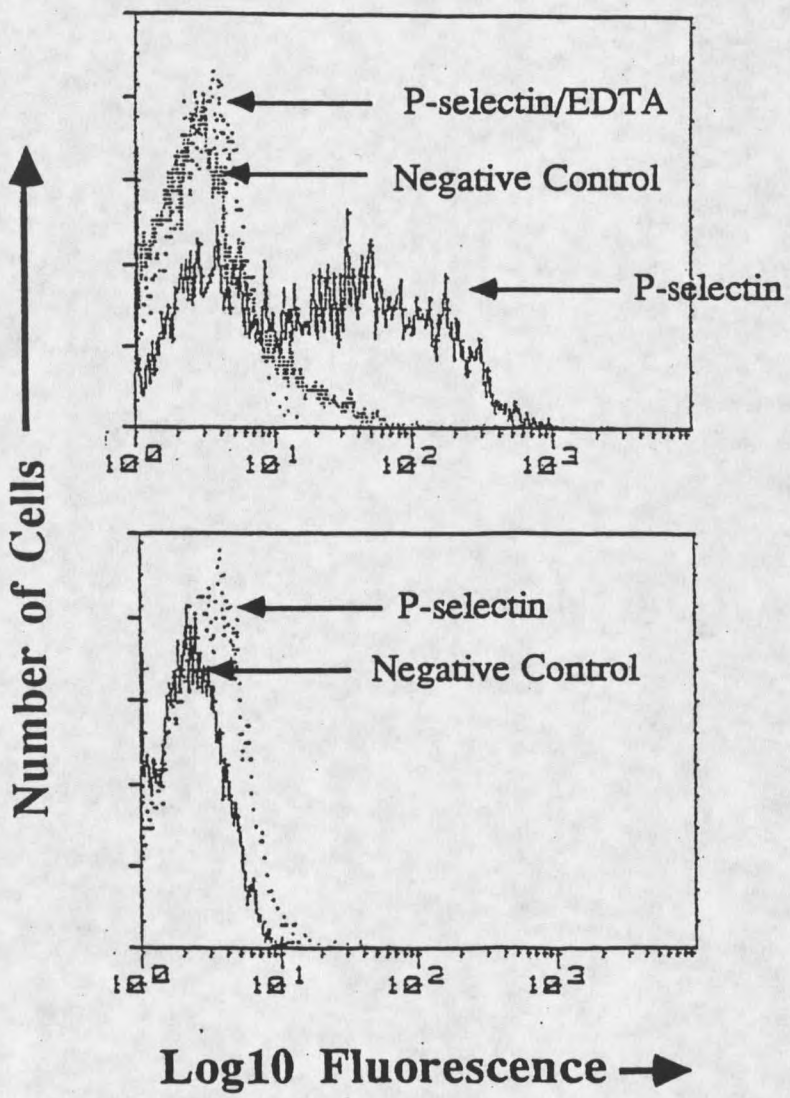


Figure 23. A P-selectin/human Ig chimera specifically stains bovine γ/δ T cells. Two-color FACS analysis staining was done on bovine mononuclear cells using the P-selectin chimera and either an anti-γ/δ T cell mAb (IL-A29) or an anti-α/β T cell mAb (CC30, anti-CD4). A live gate was set to analyze only γ/δ T cells or α/β T cells. Panels A and B show the staining of the P-selectin chimera (P-selectin) on gated γ/δ T cells and α/β T cells, respectively. Negative control is the staining with the anti-human Ig control. A similar level of background staining was seen with the CD4/Ig chimera control (data not shown). Panel A also shows that EDTA treatment blocks the staining of the γ/δ T cells by the P-selectin chimera.

To test the γ/δ T cell-P-selectin interaction in vivo, bovine skin was stimulated for 15 minutes by an intradermal injection of thrombin. The kinetics of P-selectin expression on endothelial cells is significantly different from E-selectin and other characterized inducible vascular adhesion molecules in that peak expression occurs in minutes as opposed to hours. After harvesting the injected sites, tissue sections were cut and stained for γ/δ T cells. Similar to TNF-α-inflamed skin, the thrombin-stimulated sites appeared to contain increased numbers of γ/δ T cells lining the luminal wall of dermal blood vessels. Additional analysis is obviously necessary to determine if P-selectin serves as a biologically important ligand for γ/δ T cells during inflammatory events or, if it is constitutively expressed, during normal recirculation. Furthermore, P-selectin has been shown to recognize similar carbohydrate ligands (sLe family) as E-selectin; thus the putative 250 kDa E-selectin receptor on γ/δ T cells may recognize P-selectin as well.

The in vivo studies examining γ/δ T cell accumulation in epithelial locations have primarily involved the skin due to its accessibility and ease of manipulation. Furthermore, when inflammatory events were induced, mediators, such as TNF-α, thrombin, or the bacterial-derived agents LPS and PPD, were used so that experiments can be better controlled and standardized. Live infectious agents, which are more difficult to control but induce a more biologically relevant host response, were used to a lesser degree. Preliminary studies, however, have begun to examine the primary adhesion mechanisms involved in γ/δ T cell accumulation during certain parasitic infections.

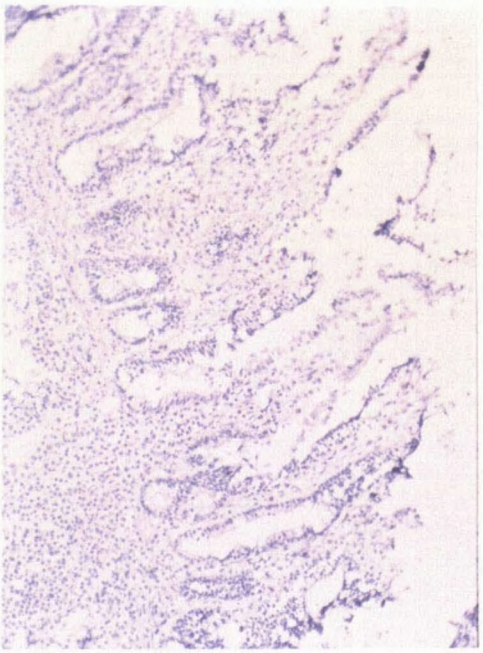
Cryptosporidium, one of several genera of protozoans in the phylum Apicomplexa, is one such infectious agent that we are currently using in the bovine system in collaboration with Mitch Abrahamson et al. In general, cryptosporidia's life-cycle takes place intracellularly within host epithelial cells of the respiratory or intestinal tract. Initiation of the life-cycle typically begins with the inhalation or ingestion of oocyts from the environment.

This is followed by excystation and epithelial cell invasion, asexual and sexual multiplication, and either termination with oocyst release via defecation or continued infection. During gut infection, cryptosporidia can be found throughout the gastrointestinal tract, though they are usually most concentrated in the ileum. Cryptosporidia within the epithelial cells appear to inflict little host cell damage visible by light microscopy, though the resulting pathological effects can be severe. In cattle, typically newborn animals, cryptosporidiosis of the gastrointestinal tract involves clinical signs such as diarrhea lasting on average 3-10 days, but is usually selflimiting in an immunocompetent host. Due to epithelial tissues being the site of cryptosporidia infection, the host-response mechanism may represent an ideal system for studying γ/δ T cell recruitment as well as effector function.

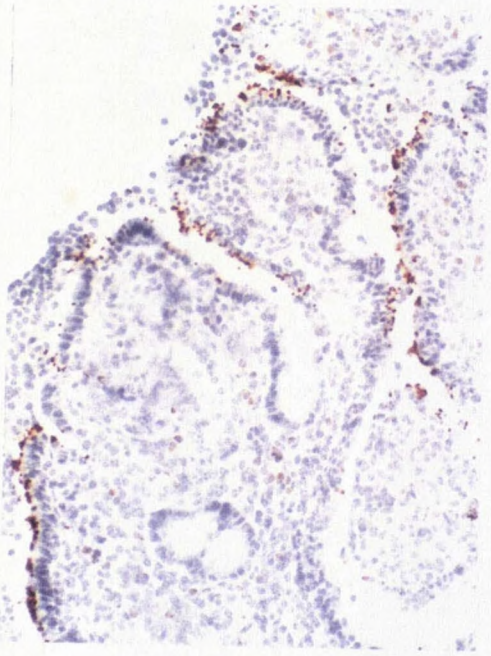
Using *Cryptosporidium parvum,* newborn calves were orally inoculated and, at days 1-13, recruitment of different lymphocyte subsets (CD4, CD8, γ/δ, and B lymphocytes) as well as vascular addressin expression were examined in the ileum. Our preliminary findings demonstrated that at day 3 of infection, CD4, CD8, and B cells were dramatically recruited into the ileal Peyer's patch (Table 7) and γ/δ T cells were recruited into the lamina propria at regions associated with parasitized epithelial cells of the mucosal villi (Figure 24-collage, and Table 7). Furthermore, unlike what we have found in normal gut, MECA-79 expression was induced within the lamina propria and the T cell regions of Peyer's patch (Figure 24-collage and Table 7); however, E-selectin expression was not observed (Table 7). By day 13, the influx of leukocytes had dissipated and ileal tissues appeared normal in their distribution of lymphocyte subsets (Table 7). Moreover, MECA-79 expression in both the lamina propria and Peyer's patch was absent (Table 7). Challenging calves several weeks later with a second dose of a *C. parvum* caused a recruitment of CD4, CD8, and B cells within the lamina propria and Peyer's patch, but not γ/δ T cells; and the expression of

C. Parvum

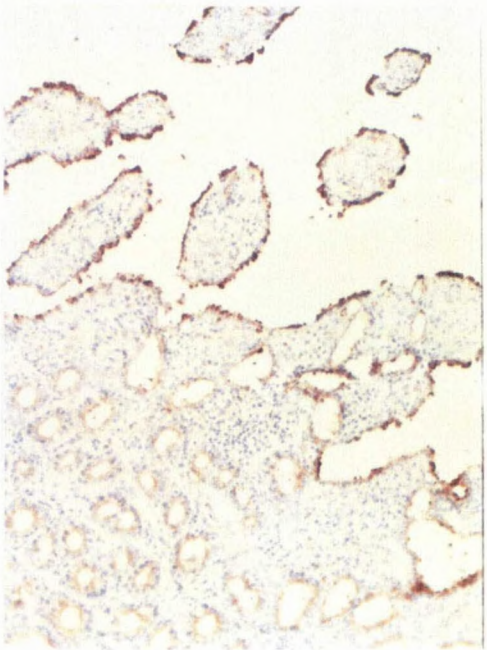
NORMAL



DAY 3, PRIMARY INFECTION

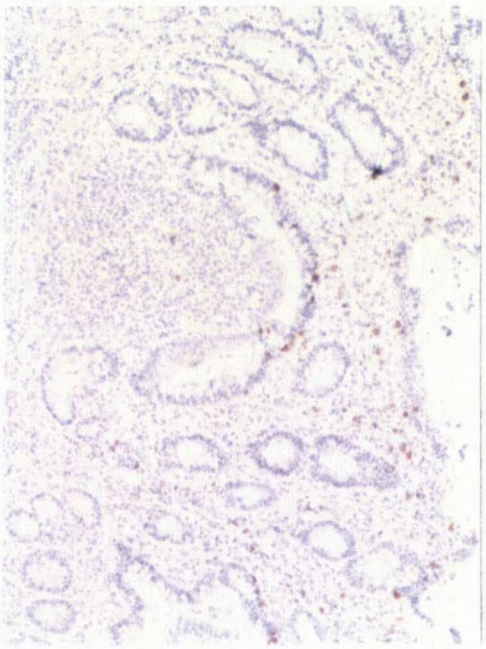


DAY 3, PRIMARY INFECTION PLUS MECA-79/DREG-56

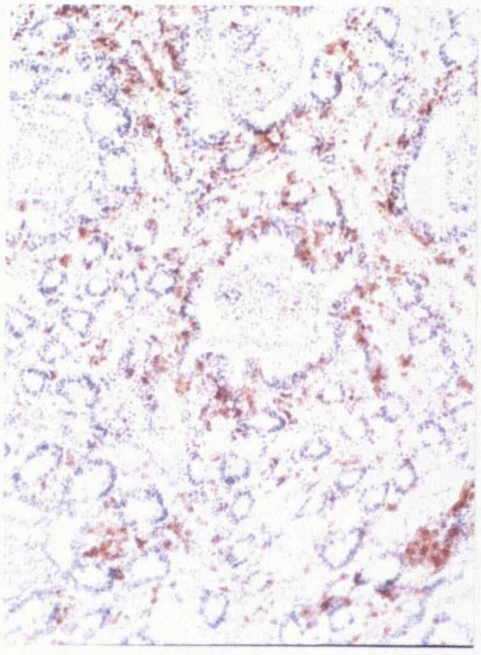


γ/δ T cells

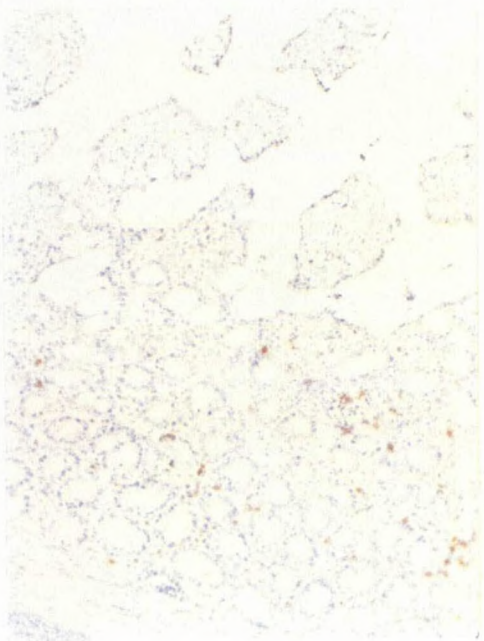
NORMAL



DAY 3, PRIMARY INFECTION

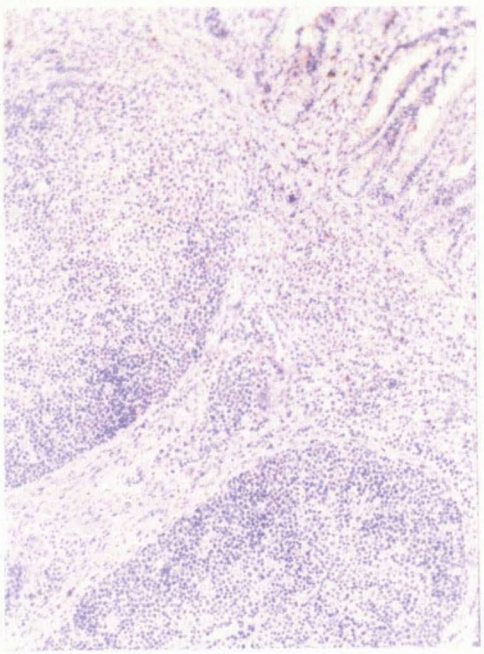


DAY 3, PRIMARY INFECTION PLUS MECA-79/DREG-56

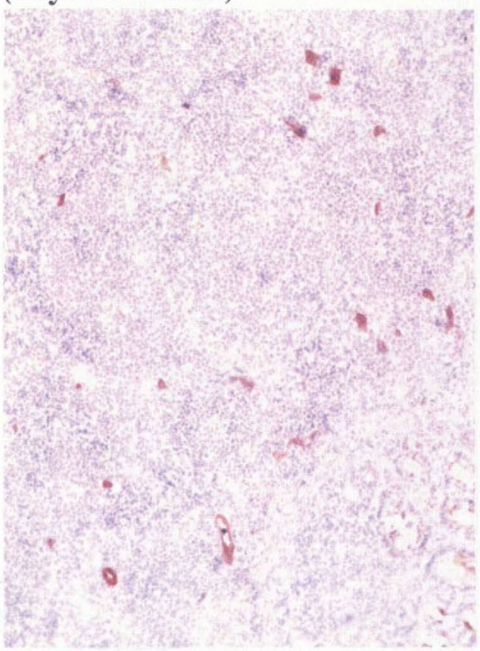


MECA-79 ANTIGEN

NORMAL



DAY 3, PRIMARY INFECTION
(Peyer's Patch)



DAY 3, PRIMARY
INFECTION
(lamina propria)

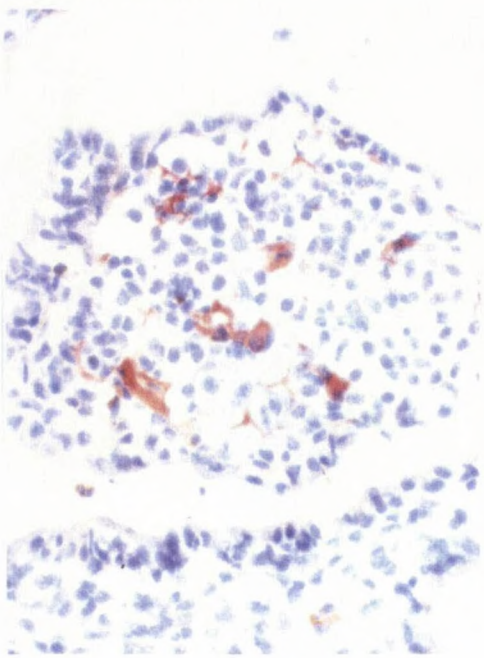


Figure 24. The administration of the mAbs MECA-79 and DREG-56 iv inhibits subsequent lymphocyte accumulation at sites of *C. parvum* infected ileum. Tissue sections prepared from normal and *C. parvum* infected bovine ileum were stained by immunoperoxidase using the mAbs; IL-A29 (γ/δ T cells), MECA-79 (MECA-79 Ag), Cp65.10 (*C. parvum*-day 3, primary infection) and Cp100.17 (*C. parvum*-day 3, primary infection following MECA-79/DREG-56 iv administration).

the MECA-79 Ag was not re-induced (Table 7).

These preliminary findings suggest that the induction of the MECA-79 Ag in the gut may be involved in the recruitment of γ/δ T cells during certain infectious diseases. However, many more experiments need to be performed, including in vivo antibody blocking of MECA-79 Ag and/or L-selectin to determine if the accumulation of γ/δ T cells can be abrogated. Indeed, this appears to be the case as demonstrated by an initial experiment. A newborn calf was first administered MECA-79 and DREG-56 (anti-L-selectin) iv, then orally inoculated with *C. parvum*. Antibody administration was repeated every 24 hours for 3 days. At day three of infection, ileal tissues were harvested, frozen, and sectioned for immunoperoxidase staining. The epithelial lining of the ileum demonstrated a typical invasion by the parasite (if not more so); however, the lymphocyte influx (Table 7), including γ/δ T cells (Figure 24-collage, and Table 7), was reduced to the level of cells found in normal tissue.

The preliminary findings described above are novel because 1) the induction of MECA-79 Ag expression has not been previously described in mucosal tissues during an infectious disease, 2) in vivo mAb blocking of a L-selectin-MECA-79 Ag-mediated recruitment of lymphocytes during infectious disease has not been previously described, and 3) the γ/δ T cell influx appears not to be associated with E-selectin expression, unlike in skin. Since γ/δ T cells express high levels of L-selectin the induction of MECA-79 may serve as a vascular addressin for these cells to more efficiently accumulate in mucosal tissues. Certain

Table 7. Distribution of lymphocyte subsets and vascular addressins in normal and *C. parvum* infected bovine ileum.

Ileal tissue	γ/δ T cells[a]	CD4 T cells	CD8 T cells	B cells	MECA-79 Ag	E-selectin
Normal						
PP[b]	+/-[c]	++	+	+++	+/-	-
LP[d]	+	+	+	+	+/-	-
Day 3, 1st infection						
PP	+/-	++++	++	+++	+++	-
LP	+++	++	++	++	++	-
Day 13, 1st infection						
PP	+/-	++	+	+++	-	-
LP	+	+	+	+	-	-
Day 3, 2nd infection						
PP	+/-	+++	++	+++	+/-	-
LP	+	+	++	++	-	-
Day 3, 1st infection, MECA-79/DREG-56 administered iv						
PP	+	++	+	+++	+/-	-
LP	+	+	+	+	-	-

a. Bovine ileal tissue sections were stained by immunoperoxidase using the mAbs; IL-A29 (γ/δ T cells), CC30 (CD4 T cells), CC58 (CD8 T cells), CC21 (B cells), MECA-79 (MECA-79 Ag), and EL-246 (E-selectin).
b. PP = Peyer's patch
c. Antibody staining distribution
d. LP = Lamina propria

secondary events, such as specific cytokine activation (explained in more detail below), may occur in mucosal tissues as apposed to PLNs, which explains why γ/δ T cells transmigrate following primary adhesion to the MECA-79 Ag.

Ongoing studies are also involved in defining the biologically important adhesion molecule(s) on γ/δ T cells that bind the vascular Selectin family members (E- and P-selectin). In Chapter 4, I described a 250 kDa molecule from γ/δ T cells that bound E-selectin with the same restrictions as γ/δ T cells. To date, we have primarily focused on generating a function-blocking mAb towards the 250 kDa molecule which we could then use in our in vitro and in vivo assays to determine if γ/δ T cell adhesion to E-selectin can be inhibited. At this time we have been unsuccessful in this attempt due to several complications: 1) using our current techniques, the 250 kDa molecule can only be isolated in microgram concentrations, which, for this molecule, is insufficient for immunizations; 2) the 250 kDa molecule appears to be highly conserved, since we have been able to isolate it from bovine, human, and mouse lymphocytes; thus, the molecule is probably nonimmunogenic in its native form. We have attempted immunizations in combination with several adjuvants [Freund's complete adjuvant, Titer Max, Gerbu, liposome inclusion (in collaboration with Jim Cutler et al.), PPD crosslinking to the 250 kDa molecule, etc.] with little success; and 3) the 250 kDa molecule appears to be heavily glycosylated (see Chapter 4), which may mask the peptide backbone from antibody recognition. We are in the process of determining the types of carbohydrate linkages (N- and/or O-linked) to the peptide backbone of the 250 kDa molecule so they can be removed using specific glycosidases to reveal additional antigenic sites for antibody recognition.

In addition to our manipulations of the 250 kDa molecule, we are immunizing animals with whole bovine γ/δ T cells (live or fixed cells in combination with various adjuvants) in hopes of generating function-blocking antibodies to the cell-surface E-selectin receptor. This approach allows for the native presentation of the 250 kDa molecule, which may be

necessary for antibody generation, but more importantly provides us with another means of generating function-blocking antibodies to the E-selectin receptor, and, thus, does not limit our approach to the 250 kDa molecule exclusively.

Using whole bovine γ/δ T cells as an immunogen in mice (BALB/c and C57BL/6), several γ/δ T cell-specific mAbs have been developed. The majority of these mAbs appear to recognize members of the WC1 family (see Chapter 1). Previously defined anti-WC1 mAbs, such as IL-A29, recognize two major isoforms and several minor isoforms by immunoprecipitation of detergent lysates from I^{125}-labeled γ/δ T cells. The two major isoforms have molecular weights of 165 kDa and 275 kDa under nonreducing conditions or 215 kDa and 300 kDa under reducing conditions. Interestingly, the 275/300 kDa WC1 isoform is similar in molecular weight to our 250 kDa (280 kDa under reducing conditions) E-selectin binding molecule. However, we have found that by standard SDS-PAGE and Western blot analysis of γ/δ T cell lysates the IL-A29 mAb stains two predominant molecules with approximate molecular weights of 165 kDa and 200 kDa (nonreducing conditions), but the 275 kDa molecule was undetectable. The lack of antibody reactivity with the 275 kDa protein may be due to the disruption of its antigenic epitopes during protein migration through the gel and/or transfer to nitrocellulose, or certain WC1 isoforms may transfer poorly to nitrocellulose. Indeed, the latter may be the case since none of our anti-WC1 antibodies appear to stain the 275 kDa molecule.

We are currently examining the 275/300 kDa WC1 isoform by immunoprecipitation of I^{125}-labeled γ/δ T cells with IL-A29 to determine if it can bind E-selectin and, if so, if it is the same molecule as our 250/280 kDa glycoprotein. Related to this pursuit, we have recently generated the mAb GD2-197.34, which specifically stains γ/δ T cells in peripheral blood (by FACS analysis) and skin or gut (by immunoperoxidase) similar to the anti-WC1 antibodies (intense cell-surface staining); however, this mAb does not react in Western blot

analysis. It is possible that this mAb recognizes the 275/300 kDa molecule. To determine this, we will immunoprecipitate the WC1 isoforms with IL-A29 and see if GD2-197.34 specifically recognizes the 275/300 kDa protein. If so, this would be unique compared to other characterized anti-WC1 mAbs, since they recognize the 165/215 kDA isoform as well. Furthermore, if the 275/300 kDa WC1 isoform binds E-selectin, GD2-197.34 could serve as a specific mAb to this molecule.

In addition to the anti-WC1 mAbs, we are currently examining two antibodies (GD3-1, GD3-5) which recognize potentially unique cell-surface molecules on γ/δ T cells. GD3-1 stained a subset of peripheral blood γ/δ T cells by FACS analysis and recognized a molecule by Western blot analysis with an approximate molecular weight of 80 kDa under nonreducing and reducing conditions. GD3-5 stained all peripheral blood γ/δ T cells by FACS analysis, and appeared to recognize a molecule with an approximate molecular weight of 160 kDa under nonreducing and reducing conditions. Both of these molecules exhibit molecular weights different from what has been described for the WC1 family. Continuing experiments are involved in further characterizing these molecules. Such lineage-specific molecules may play a role in the unique functional capabilities of γ/δ T cells.

Future directions

Previous studies using myeloid cells have revealed that leukocyte extravasation is a complex process involving at least three steps which represent an association of signalling events (by cell-cell interactions and various cytokines) and adhesion events (by primary and secondary adhesion proteins) that together function as a combinational process resulting in

very specific tissue and site extravasation by leukocytes. The steps are 1) primary adhesion, 2) secondary adhesion, 3) transmigration. Recently, through manipulation of signalling cascades and adhesive mechanisms, lymphocyte migration into certain tissues has been shown to involve multiple steps as well. The experimental findings described in this thesis provide further support for the model.

The tissue-selective mechanisms used by bovine γ/δ T cells provide the first biological demonstration of the three-step model for lymphocytes. As described above, primary adhesion by γ/δ T cells to the vascular wall in certain tissues occurs via a particular vascular addressin (MECA-79 Ag, E-selectin, P-selectin) and homing receptor (L-selectin or the putative 250 kDa receptor) combination. However, this adhesion event alone does not dictate whether these cells will transmigrate into the underlying tissue, as elegantly demonstrated in the PLNs. γ/δ T cells accumulate along the vascular wall of PLN PCV via L-selectin and the MECA-79 Ag, respectively, but these cells do not transmigrate into the underlying tissue as α/β T cells do. However, this same adhesion event appears to direct γ/δ T cells to the ileum during certain inflammatory conditions where these cells do extravasate into the underlying tissue in large numbers.

The events which determine whether γ/δ T cells will enter the tissue appear to occur in the latter steps following primary adhesion, which include de-adhesion of the primary binding event and activation of secondary adhesion proteins for tight adhesion and extravasation. Since γ/δ T cells express important secondary adhesion proteins for extravasation, the necessary agent that induces their transmigration into the inflamed ileum, as apposed to the PLNs, may be a specific activating and/or chemotactic factor. These inflammatory mediators consist of a variety of cytokines including a newly described family referred to as the chemokines, which induce integrin-mediated adhesion and migration of a number of leukocyte subsets. The various chemokine family members are receiving a lot of

attention due to their ability to induce select leukocytes within specific tissues. In addition, certain chemokines can adhere to the vascular wall (via proteoglycans) where they are presented to the leukocytes that are initially interacting with the endothelium via the primary adhesion process.

Future studies will further characterize the adhesion molecules which direct γ/δ T cells to sites of inflammation and epithelial locations, as well as attempt to define the role these activating/chemotactic factors play in γ/δ T cell tissue-selective trafficking.

Some of the experimental findings described above are included in the following publications:

1. **Walcheck, B.** and Jutila, M.A. 1994. Bovine γ/δ T cells express high levels of functional peripheral lymph node homing receptor (L-selectin). Int. Immunol. 6:81.

2. **Walcheck, B.**, Watts, G. and Jutila, M.A. 1993. Bovine γ/δ T cells bind E-selectin via a novel glycoprotein receptor: Characterization of the first lymphocyte-E-selectin interaction in an animal model. J. Exp. Med. 178:853-863.

3. **Walcheck, B.**, White, M., Kurk, S. and Jutila M.A. 1992. Characterization of the bovine peripheral lymph node homing receptor: A lectin cell adhesion molecule (LECAM). Eur. J. Immunol. 22:469-476.

4. **Walcheck, B.**, Palencanda, A. and Jutila, M.A. 1993. Analysis of lymphocyte migration in vivo. In "Lymphocyte adhesion molecules", Shimizu, Y. editor. R.G. Landes Company, p173-190.

5. **Walcheck, B.** and Jutila, M.A. 1993. Regulation of leukocyte entry into inflamed tissue: Role of the peripheral lymph node homing receptor (L-selectin). In "Structure and function of molecules involved in leukocyte adhesion", Faanes, R.B., Kishimoto, T.K., Lipsky, P., Smith, C.W. and Rothlein, R. editors. Springer-Verlag, New York, p 182-190.

6. Bargatze, R.F., **Walcheck, B.**, Kurk, S., Watts, G., Butcher, E.C., and Jutila, M.A. 1993. Lymphocyte tissue-specific recruitment from the blood: A complex multi-step process. Transplantation Science. 3:155-160.

7. Jutila, M.A., Watts, G., **Walcheck, B.** and Kansas, G.S. 1992. Characterization of a functionally important and evolutionarily well-conserved epitope mapped to the short consensus repeats of E-selectin and L-selectin. J. Exp. Med. 175:1565-1573.

8. Palencanda, A., **Walcheck, B.**, Bishop, D.K., and Jutila, M.A. 1992. Rapid activation-independent shedding of LECAM-1 induced by cross-linking agents. Eur. J. Immunol. 22:1279.