Demonstration of the leukocyte-endothelial cell adhesion cascade involved in lymphocyte recirculation and neutrophil and lymphocyte localization to sites of inflammation
by Robert Frost Bargatze

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology
Montana State University

Abstract:
Experimental evidence derived from multiple experimental systems, presented in this thesis, support the hypothesis that tight leukocyte adhesion to endothelial cells (EC) is regulated by a multi-step process that includes rolling, up-regulation of secondary adhesion molecule affinity through signaling, and permanent arrest. Developing an in vivo video microscopy analysis system, I have shown that mouse lymphocytes exhibit a multi-step behavior of binding when interacting with Peyers patch (PP) high endothelial venules (HEV). Also using this system, I have identified the molecules responsible for the multi-step lymphocyte PP-HEV rolling and adhesion behaviors: L-selectin mediates rolling, α4β7 integrin mediates slowing of rolling and activation dependent adhesion, LFA-1 may play a role in α4β7 activation, and MadCAM on the HEV acts as a ligand for both L-selectin and α4β7. Through developing an in vitro shear system to study human peripheral blood lymphocytes (PEL) interactions with E-selectin transfectants and human umbilical vein endothelial cells (HUVECs), I have demonstrated that E-selectin can support L-selectin-independent lymphocyte rolling. HUVECs in this system can support rolling through E-selectin and all aspects of the in vivo three-step binding process. Also using this system I examined the effects of chemokine signaling on lymphocyte adhesion and defined a new mechanism where MIP-lβ and IP-10, in minutes, induce increased avidity through selectins, slowing lymphocyte rolling. Further, I have shown in vitro that human neutrophils roll on E-selectin transfectants and HUVEC and defined the molecular basis through functional blocking with a unique mAb, EL-246, that acts on both L- and E-selectin impeding their adhesive functions. Finally, in vitro I have demonstrated a new mechanism for neutrophil localization where neutrophils roll on pre-adhering neutrophils augmenting their continued recruitment.

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I dedicate this thesis to my grandmothers and grandfathers:
Anna Frost and Alex C. Frost and Katie Bargatze and Robert C. Bargatze;
for imparting to me the gift of curiosity for exploring the natural universe
and an unending desire to understand its depths.

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ABSTRACT

Experimental evidence derived from multiple experimental systems, presented in this thesis, support the hypothesis that tight leukocyte adhesion to endothelial cells (EC) is regulated by a multi-step process that includes rolling, up-regulation of secondary adhesion molecule affinity through signaling, and permanent arrest. Developing an *in vivo* video microscopy analysis system, I have shown that mouse lymphocytes exhibit a multi-step behavior of binding when interacting with Peyers patch (PP) high endothelial venules (HEV). Also using this system, I have identified the molecules responsible for the multi-step lymphocyte PP-HEV rolling and adhesion behaviors: L-selectin mediates rolling, $\alpha 4\beta 7$ integrin mediates slowing of rolling and activation dependent adhesion, LFA-1 may play a role in $\alpha 4\beta 7$ activation, and MadCAM on the HEV acts as a ligand for both L-selectin and $\alpha 4\beta 7$. Through developing an *in vitro* shear system to study human peripheral blood lymphocytes (PBL) interactions with E-selectin transfectants and human umbilical vein endothelial cells (HUVECs), I have demonstrated that E-selectin can support L-selectin-independent lymphocyte rolling. HUVECs in this system can support rolling through E-selectin and all aspects of the *in vivo* three-step binding process. Also using this system I examined the effects of chemokine signaling on lymphocyte adhesion and defined a new mechanism where MIP-1ß and IP-10, in minutes, induce increased avidity through selectins, slowing lymphocyte rolling. Further, I have shown *in vitro* that human neutrophils roll on E-selectin transfectants and HUVEC and defined the molecular basis through functional blocking with a unique mAb, EL-246, that acts on both L- and E-selectin impeding their adhesive functions. Finally, *in vitro* I have demonstrated a new mechanism for neutrophil localization where neutrophils roll on pre-adhering neutrophils augmenting their continued recruitment.

CHAPTER 1

INTRODUCTION

The Leukocyte-Endothelial Cell Adhesion Cascade

The interactions of leukocytes with endothelium, which facilitate their blood-borne recruitment to sites of inflammation and lymphoid organs, are a critical step in regulating the distinct but closely related processes of inflammation and immune host surveillance. These defensive processes have evolved in parallel to provide an effective means for overlapping host protection. Leukocyte surveillance can protect against infection, act as a vigil for neoplastic conversions, employ mechanisms to recognize and reject foreign tissues, and, unfortunately in some cases, result in overt pathology. The unravelling of the molecular mechanisms of endothelial-leukocyte recruitment is brief, spanning only one-and-a-half decades. Even so, these recent advances in understanding the processes and molecular basis of leukocyte-endothelial cell recruitment have spurred vigorous inquiry into developing novel therapeutics and approaches for treating many age-old immune/inflammatory diseases.

The first event required for leukocyte entry into the body's tissues from the circulating blood, is recognition and adhesion to the surface of the blood vessel wall. A strong functional paradigm has been developed to describe leukocyte trafficking and consists of a three step process for

leukocyte-endothelial cell binding. Work conducted for this thesis has led directly to the generally accepted theory that during homing and inflammatory recruitment, lymphocytes, like neutrophils, employ this three-step process of recognition leading to strong endothelial cell adhesion. The three step model requires the leukocyte to make a very rapid (1.5-2.0 sec) and accurate assessment as to whether it should bind to the endothelium and transmigrate into a tissue after only a brief encounter with the endothelial-cell surface on the vessel wall. This assessment is based on endothelial-cell presented leukocyte adhesion molecules and molecular signaling information in either soluble or attached form [Tanaka et al., (1); van Kooyk et al., (2)]. In both cases, the molecules reveal critical information on the condition of the underlying tissue.

The experimental evidence supporting the multi-step hypothesis is significant and has been endorsed in many independent studies [reviewed in (3-8)]. The first event to occur in the temporal sequence is the collision of leukocytes with the blood-vessel wall. These collisions, a consequence of blood-vessel morphology and hemodynamic forces, can productively lead to leukocyte-endothelial cell rolling or in the return of the cell to the circulation. Rolling occurs only after tethering (contact) of leukocyte shear-capable adhesion molecules with their counter ligands on the endothelium, as illustrated in Figure 1, and can be mediated by separate leukocyte molecules. These tethering and rolling interactions can be mediated by both adhesive molecules identified as "selectins" as well as the $\alpha 4\beta 7$ and $\alpha 4\beta 1$ integrins. These molecules slow the cell from the blood flow allowing an "adhesokine" (an adhesion molecule signaling factor that induces increased adhesion molecule avidity and/or numbers) to bind its leukocyte receptor.

The binding of this effector can trigger a transmembrane signal, the second step, resulting in the induction of adhesion activity by an additional class of adhesion receptor, the leukocyte integrins, and in some cases increases their numbers on the leukocyte surface (Figure 1). If the endothelium presents a counter ligand for the signal-induced integrin adhesion molecules, the cell may be further slowed to where it arrests on the endothelial surface, completing the third step. The leukocyte may at this point go on to strengthen its adhesive hold on the endothelium via morphology changes and engagement of low-to-non-shear-acting integrin adhesion receptor pairs that promote strong adhesion, motility, and transmigration into the underlying tissue to follow chemotactic signals (Figure 1). Alternatively, the leukocyte may choose to release from the endothelium and return to the blood. This three step model of cell adhesion can be descriptively termed a "leukocyte-endothelial cell adhesion cascade."

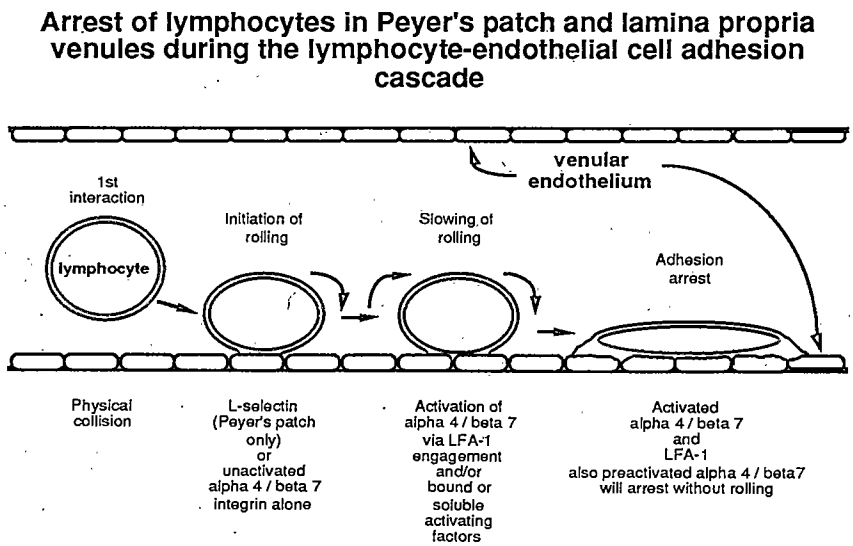


Figure 1. Events of the leukocyte-adhesion cascade, shown specifically for

mucosal binding lymphocytes. The lymphocyte enters from the blood flow, colliding with the blood-vessel wall and initiating rolling via L-selectin mediated-tethering to MadCAM. During rolling, $\alpha 4\beta 7$ integrin slows rolling, again via MadCAM ligation, as it becomes activated via signaling interactions of LFA-1 or endothelial cell-presented "adhesokines" that engage their leukocyte cell-surface receptors. As the full potential of adhesion avidity is reached by greater numbers and/or higher affinity of $\alpha 4\beta 7$ interactions, the lymphocyte is brought to a full arrest. Cells possessing preactivated $\alpha 4\beta 7$ can arrest on mucosal venules without L-selectin participation with little rolling before arrest.

Completion of each stage of the three-step process is required for successful strong adhesion of the leukocyte to the endothelium. Since each step is a unique recognition event, specificity can reside at any or all points in the cascade. Furthermore, each step may be mediated by many receptor-ligand pairs; hence, the number of potential leukocyte-endothelial cell specificities is the product of diversity at each of these recognition steps. Thus, selective recruitment can be very specific and plastic, recognizing the unique tissue-associated aspects of the vessel beds of lymphoid organs, or alternatively, remarkably responsive to inflammatory-induced molecular changes on the endothelium occurring to combat infection, disease, or trauma.

Constitutive lymphocyte recirculation and tissue-selective localization.

The first leukocyte tissue-selective adhesion system to be studied was the homing of lymphocytes to lymph nodes and Peyers patches which

occurs during constitutive recirculation and immune surveillance. In the early 80's, leukocyte adhesion receptors for the endothelium were theorized to have tissue-specific binding properties. This hypothesis was first based on lymphocyte recirculation data generated in rat [Gowans and Knight, (9)] showing that mucosal and peripheral-derived lymphocytes, labeled with radioactive tracers, followed distinct recirculation patterns that generally returned them to their lymphoid tissues of origin. In the late seventies and early eighties, studies showed that there are distinct lymphocyte subsets that have unique lymph-node high-endothelial venule (HEV) binding properties.

HEV are specialized postcapillary venules, comprised of rounded "high" or plump endothelial cells, that are present in lymph nodes and at sites of chronic inflammation. Lymphocyte-HEV binding competence correlates well with lymphocyte development and differentiation. Germinal center B cells and cortical thymocytes are sessile immature cells that do not bind HEV in vitro [Reichert et al., (10); Butcher et al., (11)]. Alternatively, recirculation competent, mature resting lymphocytes are capable of HEV binding in all lymphoid tissues; however they are composed of subsets, dependent on tissue origin, that show a general preferential binding for peripheral lymph nodes (PLN) or Peyer's patch (PP) HEV [Stevens et al., (12)]. The tissue-selective hypothesis was more directly shown in work by Butcher, Scollay and Weissman (13), where mouse lymphoma cell lines were isolated and shown to preferentially adhere to the HEV of PLN or PP. This was later shown to be the case for human

lymphomas, with the addition of the identification of lymphomas specific for inflamed joint synovial venules [Jalkanen et al., (14)]. These studies employed the ex vivo lymphocyte-HEV binding assay developed by Stamper and Woodruff (15) and for the first time demonstrated endothelial-selective binding. Lymphoma phenotypes were characterized that showed peripheral, mucosal, dual, and non-binding phenotypes. The mouse-derived lymphomas, exhibiting each of the specific binding phenotypes from these studies, were tested in vivo. We found that HEV-binding, but not HEV-non-binding, lymphomas spread metastically via the blood, suggesting that homing receptors are a key component in lymphoid metastasis [Bargatze et al., (16)]. Collectively, these findings strongly support the hypothesis for "tissue-specific" lymphocyte adhesion receptors for HEV and intensified interest in their identification and characterization.

L-selectin: The lymphocyte PLN homing receptor.

The first "tissue-specific" lymphocyte adhesion receptor to be characterized was phenotypically and functionally identified by the monoclonal antibody (mAb) MEL-14. Gallatin et al. (17) demonstrated that MEL-14 recognizes a lymphocyte surface glycoprotein, now known as L-selectin. In this first paper describing the physical and functional properties of L-selectin, it was clearly demonstrated that MEL-14, both in vitro and in vivo, almost completely inhibits lymphocyte binding or homing to PLN HEV. (This paper also represented my first effort in leukocyte adhesion biology; the in vivo data coming from my experiments.) Rasmussen et al.

(18) produced a mAb against rat PLN homing receptors that has function-blocking properties similar to MEL-14, but the molecule(s) it sees was never fully characterized. Also in the mid 80's, L-selectin was found to be expressed on the surface of other leukocytes, including neutrophils, monocytes, and eosinophils. In the study by Lewinsohn et al. (19), we demonstrated that MEL-14 would block neutrophil binding to PLN HEV and their recruitment to acute inflammatory sites in a mouse model. It is now known that L-selectin is expressed on all leukocytes except for a population of lymphocyte memory cells [Lewinsohn, (19); Stoolman, (20)].

The 80-95 kD MEL-14-defined L-selectin molecule functions as an animal lectin, blockable by phosphomannan competitors, capable of binding unique carbohydrate determinants on PLN HEV [Yednock et al., (21)]. Cloning of L-selectin by Siegelman et al. (22) and Lasky et al. (23) revealed striking homology to the C-type mammalian lectins in the L-selectin N-terminal domain. The L-selectin "Drickamer motif" lectin domain [(23); Watson et al., (24)] exhibits a typical strict calcium dependence for carbohydrate recognition, which is the precise cation requirement for lymphocyte-PLN HEV binding. Thus, the rolling/binding specificity of L-selectin for endothelial ligands is believed to reside largely in this domain; however, an epidermal growth factor (EGF) domain that is contiguous with the lectin domain expresses a mAb blockable epitope that contributes to the rolling/binding function of the molecule. The remaining structure of L-selectin is composed of two identical repeats of a complement binding protein-like domain (SCRs), a transmembrane domain, and a short cytoplasmic tail containing one potential phosphorylation site. The same

general domain structures are present in the other members of the selectin family: E-selectin on endothelium and P-selectin on endothelium and platelets [Bevilacqua et al., (25); Johnston et al., (26)]. These molecules are specifically involved with inflammation-driven, blood-borne recruitment of leukocytes to acute and chronic inflammatory sites of the body.

Evaluating the blood-borne spread of murine lymphomas again led us to a new finding; L-selectin could support adhesion to PP HEV [Bargatze et al., (27)]. This seemed to conflict with earlier findings that L-selectin mediated binding to PLN but not PP HEV. Now, in the context of a three-step adhesion model, it is clear that L-selectin mediates only the tethering/rolling interaction with the mucosal endothelium that leads to specific integrin-mediated tight adhesion. Hamann et al. (28) confirmed these observations in vivo showing a 40-50% reduction of homing to mouse PP when lymphocytes were treated with the anti-L-selectin mAb MEL-14.

The peripheral node addressin is a functional receptor for lymphocytes on HEV.

Identification of the PLN ligand for L-selectin on the HEV was the focus of intense effort soon after L-selectin and its specificity for binding were identified. It was reasoned that if a specific leukocyte counter-receptor for PLN HEV was required for binding then an equally specific receptor expressed on the endothelium must exist. Using the mAb MECA-79, Streeter et al. (29) were rewarded with the identification of a group of PLN HEV-expressed glycoproteins which they named peripheral lymph node

addressin (PNAd). Functional characterization of the MECA-79 mAb using the HEV binding assay and homing of radiolabeled lymphocytes in vivo showed that it blocks HEV binding and PLN localization of lymphocytes in a manner identical to MEL-14, though less efficiently. Work done by Berg et al. (30) showed that the MECA-79 epitope is apparently a carbohydrate antigen presented on the PNAd glycoprotein and that this epitope is critical for supporting lymphocyte adhesive interactions.

More recently, MECA-79 was used to purify the PNAd for in vitro assays. Berg et al. (31) demonstrated that the isolated PNAd supports binding of lymphocytes which is L-selectin dependent. Also, the ability of MECA-79 isolated PNAd to support lymphocyte and neutrophil rolling under shear was tested in vitro. Glass capillary tubes were coated with isolated PNAd and individually integrated into a closed silicone tubing loop attached to a peristaltic pump. Neutrophils or lymphocytes in tissue culture media were infused into the loop, flow was started to simulate blood flow, and events occurring in the capillary tube were recorded by video microscopy (Figure 2). This system demonstrated that lymphocytes and neutrophils roll on the isolated PNAd molecules exhibiting behaviors very similar to those observed for these cell types in vivo (Bargatze, Berg, Palecanda, Butcher, and Jutila, unpublished observations).

Subsequent studies have demonstrated that the PLN addressin is induced in postcapillary venules at diverse sites of the body and is found in association with chronic inflammation [Michie et al., (32); Hanninen et al., (33)], and autoimmune disease [Berg et al., (34)]. In fact, using the L-selectin-positive PLN HEV-binding lymphoid tumors, I found that MECA-79 defined

an L-selectin ligand expressed on PP HEV at low but functional levels (27). Berg et al. (35) confirmed that the MECA-79 epitope, expressed on in vivo isolated mucosal addressin (MadCAM, which supports lymphocyte trafficking to PP) supports L-selectin-dependent rolling in the capillary tube shear system. These reports have led directly to the studies reported in Chapter 4, in which L-selectin-dependent rolling on MadCAM in vivo is demonstrated to support the tethering of lymphocytes to PP HEV, the first step in the recruitment process for lymphocyte recirculation in mucosal immune surveillance.

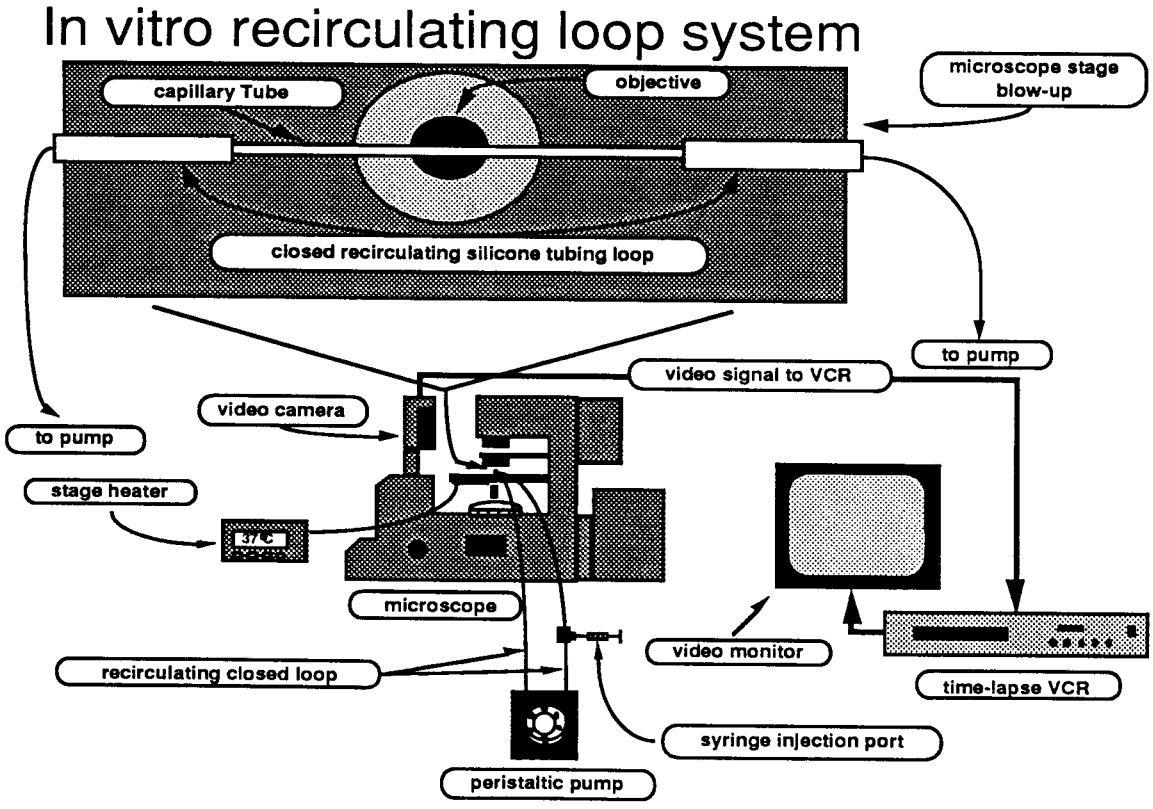


Figure 2 To examine leukocyte adhesion (tethering-rolling, slowing, and

permanent arrest) in a controlled in vitro setting I developed the novel capillary tube, closed-loop, shear assay system as depicted above. This system provides the means to make measurements of the interaction of suspended cells with immobilized substrates, such as endothelial cells, transfected fibroblasts, marginating leukocytes, and purified adhesion proteins, under conditions reflective of those seen under blood flow.

Identification of a mucosal-specific lymphocyte homing receptor.

The recognition of PP-specific HEV-binding lymphomas suggested that, as with the peripheral HEV system, mucosal-specific homing receptors for MadCAM must exist. Again, several groups reported early findings suggesting the identification of such a receptor for lymphocytes. Chin et al. (36) produced a mAb that blocks rat lymphocyte binding to PP HEV, as did Jalkanen et al. (37). The molecule identified by Chin et al. was never fully characterized; the molecule identified by Jalkanen has been shown by cDNA cloning [Golstein et al., (38)] to be a lymphocyte-specific form of CD44 involved with adhesion, but not to be the central player in mucosal lymphocyte trafficking. In the first study to examine the cell-surface phenotype of gut intraepithelial leukocytes (IEL), Schmitz et al. (39) reported that these cells lacked expression of L-selectin, as assessed by MEL-14 mAb staining. They also discovered that these cells avidly bound PP HEV in contrast to PLN HEV, where IEL showed a near background level of binding. These experiments clearly demonstrated that a population of leukocytes, IEL, could demonstrate exclusive tissue selectivity for their PP versus PLN homing phenotype.

A mucosal homing receptor breakthrough occurred in 1989 with the production of the R1-2 monoclonal antibody [Holtzmann et al., (40)]. This antibody recognizes the integrin heterodimer α4β7 expressed on mouse lymphocytes with function distinct from the previously characterized VCAM-1 binding integrin α4β1 (40). Holtzmann et al. demonstrated that lymphocyte binding to PP HEV in vitro could be specifically blocked by R1-2, showing this interaction to be distinct from that occurring in PLN. Unfortunately, this mAb was not effective in blocking lymphocyte HEV interactions in vivo, so confirmation of this interaction under physiological conditions waited until Issekutz (41) showed that α4 in the rat and later Hamann et al. (42) showed that α4 as well as β7 in the mouse was required for in vivo homing to mucosal lymphoid tissues.

Interestingly, α4β7 has also recently been demonstrated to bind VCAM-1 and fibronectin in static assays [Postigo et al., (43)]. We have confirmed VCAM-1 binding under physiological shear (Berlin et al. in press, Cell), with data strongly suggesting that α4 is the primary chain of the heterodimer supporting the specificity.

The mucosal addressin directs lymphocyte traffic to gut HEV and lamina propria venules.

During our initial quest for tissue-specific ligands expressed on HEV for lymphocytes, coinciding with the characterization of the PNAD mAb MECA-79 we found a new mAb that specifically stained PP versus PLN HEV. This antibody, MECA-367, blocks binding in vitro and homing in vivo of

mucosal-specific lymphomas and normal lymphocytes Berg et al. (30) and was used to characterize the mucosal addressin glycoprotein (MadCAM). Using a non-blocking mAb to MadCAM (MECA-89) in an immunoaffinity column, the MadCAM molecule was isolated from mucosal lymphoid vessels and immobilized in planar lipid membranes. Both mucosal-specific lymphomas and normal lymphocytes bound the isolated MadCAM in static assays, and binding was blocked by treatment with MECA-367.

MadCAM was recently cloned by Briskin et al. (44), revealing an unexpected and unique domain structure. The N-terminus of MadCAM presents a domain with a structure and sequence similar to both ICAM-1 and VCAM-1 which is followed by a VCAM-1 like domain. It is likely these domains interact with $\alpha 4\beta 7$ integrin on lymphocytes, facilitating adhesion (44). These regions are followed by a mucin-like domain which may facilitate lymphocyte rolling in PP HEV (44). The final domains, include an IgA-like domain, a single membrane-spanning segment and cytoplasmic tail. Much of the behavior observed for lymphocyte-HEV interactions can be extrapolated from the properties of MadCAM based upon this diverse presentation of defined adhesion molecule-related structures. It is interesting to note that MadCAM can be induced on endothelial cells in culture [Sikorski et al., (45)], while PNAD has not yet been successfully expressed in vitro.

Most recently, we have shown that MadCAM, when isolated from mucosal lymphoid venules, supports lymphocyte rolling adhesion in the in vitro capillary tube (shown in Figure 2) to examine leukocyte shear-dependent interactions [Berg et al., (46)]. These results provide a specific

identification for the molecular basis of the L-selectin binding to PP HEV which we reported earlier (27). Interestingly, MECA-79, the mAb recognizing PNAD, identifies a subset of MadCAM that supports L-selectin-dependent rolling (46). This carbohydrate epitope, which can decorate MadCAM, appears to be the critical adhesive binding/recognition element for L-selectin. Thus, expression of this epitope can be induced in non-peripheral locations directing peripheral lymphocyte trafficking to mucosal sites.

In a manuscript just accepted for publication (Berlin et al., Cell, in press), we have again used the in vitro shear system to show that MadCAM, under shear, can support the binding of lymphocytes and lymphomas requiring only activated $\alpha4\beta7$. We also confirmed these observations in vivo in gut lamina propria venules where there is no L-selectin component of adhesion. There again, $\alpha4\beta7$-MadCAM interactions are primarily responsible for supporting the lymphocyte/lymphoma blood-borne localization (Berlin et al., Cell, in press). Thus, it appears that on the HEV of the Peyer's patch, MadCAM acts as a constitutive ligand that mediates lymphocyte-selectin rolling and facilitates strong integrin adhesion to support the lymphocyte homing process. In gut lamina propria venules, MadCAM and activated $\alpha4\beta7$ can arrest circulating lymphocytes in the absence L-selectin, stopping them after a very short $\alpha4\beta7$ mediated rolling interaction with the endothelium. This is of particular interest since it demonstrates that a single integrin-mediated adhesive step can compress the multistep process into one event leading to lymphocyte permanent arrest on the endothelium.

LFA-1 is a non-tissue-specific participant in lymphocyte binding to HEV.

Lymphocyte interactions with HEV during static assay conditions have been known since the late 1980s to be supported by the integrin LFA-1 [Hamann et al., (47)]. These adhesive interactions, however, are not tissue selective, and mAbs only block lymphocyte HEV binding approximately 40 percent on either PP and PLN HEV. Interestingly, LFA-1 also represents a molecule involved in both receiving and delivering activating signals [Binnerts et al., (48)]. LFA-1 is a versatile adhesion molecule capable of interacting with ICAM-1 on lymphocytes, epithelial cells, and inflamed endothelium; ICAM-2 on the vascular endothelium and some lymphoid cells; and ICAM-3 only on leukocytes (48). ICAM-2 is believed most likely to be involved in the recirculation of resting lymphocytes as it is the predominant ICAM form expressed on resting endothelium [de Fougerolles and Springer, (49)]. Crosslinking of T-cell surface receptors, such as CD2 and CD3, or addition of the phorbol ester PMA generates intracellular signals that lead to increased avidity of LFA-1 for ICAM-1, resulting in stronger lymphocyte adhesion to the endothelium [Dustin and Springer, (50); van Kooyk et al., (51); , Rothlein and Springer, (52)]. It also was discovered that a mAb-binding LFA-1 could similarly alter its adhesive properties inducing tighter LFA-1-ICAM-1 binding (48). Interestingly, engagement of LFA-1 and its ligand ICAM-3 can also act to deliver a co-stimulatory signal for both resting and activated T-cells [Hernandez-Casselles et al., (53); Campanero et al., (54)]. These diverse adhesion and signaling properties suggest that LFA-1

on lymphocytes may play a multifunctional role in the lymphocyte-endothelial cell adhesion cascade that involves regulation of other adhesion receptors through receiving and delivering intracellular signals, as well as directly supporting HEV binding.

Lymphocytes, signal transduction, and the three-step model of leukocyte binding.

When I started my thesis work in 1990, a muti-step hypothesis for localization of leukocytes to endothelium during lymphocyte recirculation or to sites of inflammation had yet to be formulated. The adhesion receptors that support the process had been largely identified and characterized, but their physiological function and temporal role in the blood-borne adhesion process had not been accurately determined. The interactions of lymphocytes with the PP HEV of mice [Bjerknes et al., (55); (16)] and neutrophils in mesenteric venules of rabbits [Arfors et al., (56)] had been observed and described in detail in vivo to show a diversity of rolling and sticking behaviors. Dillon et al., (57) had demonstrated that platelet activating factor (PAF) could promote tight adherence of leukocytes to endothelium. LFA-1 was shown to be capable of activation to support tight adhesion to endothelium possibly through factors, such as PAF. All of these were critical elements in the multi-step process yet to be discovered. In 1989, a key observation by Huang et al. (58) demonstrated that pertussis toxin (Ptx) treatment, which interrupts transmembrane signaling, is effective in preventing lymphocyte localization to HEV-bearing lymphoid organs. This

finding suggested a requirement for signal transduction in the homing process. The stage was set for unravelling the intricate details of the leukocyte-endothelial cell adhesion cascade. The model was first presented in a two-step form by our group at Stanford in 1991 [von Andrian et al. (59)].

With these initial observations, Kishimoto (60) proposed a dynamic three-step model for neutrophil localization to inflammatory sites and soon after Butcher (61) proposed a generalized model for leukocytes predicting that a three-step model would also hold for lymphocyte localization to HEV during constitutive recirculation. It was at this point in early 1991 that we began to evaluate the in vivo interaction of lymphocytes in PP HEV (Figure 3). We used Ptx and PMA to determine if lymphocytes required a functional Ptx-sensitive adhesion-strengthening signaling pathway, as suggested by Huang et al. (58) to arrest on PP HEV. The results of these experiments were recently published (16) and are described in detail in Chapter 2 of this thesis. The findings showed that lymphocytes, like neutrophils, required multiple adhesion steps to arrest on the endothelium.

The data describing the identification of the adhesion receptor pairs responsible for the behavior of lymphocyte binding to PP HEV are contained in Chapter 3 of this thesis and has recently been submitted for publication. In this extensive in vivo study, we evaluated each step in the rolling, slowing, and tight adhesion steps of lymphocyte binding to HEV with adhesion receptor-specific function-blocking mAbs. Our evaluation showed that lymphocyte-HEV tight adhesion could be blocked by mAbs directed against LFA-1.

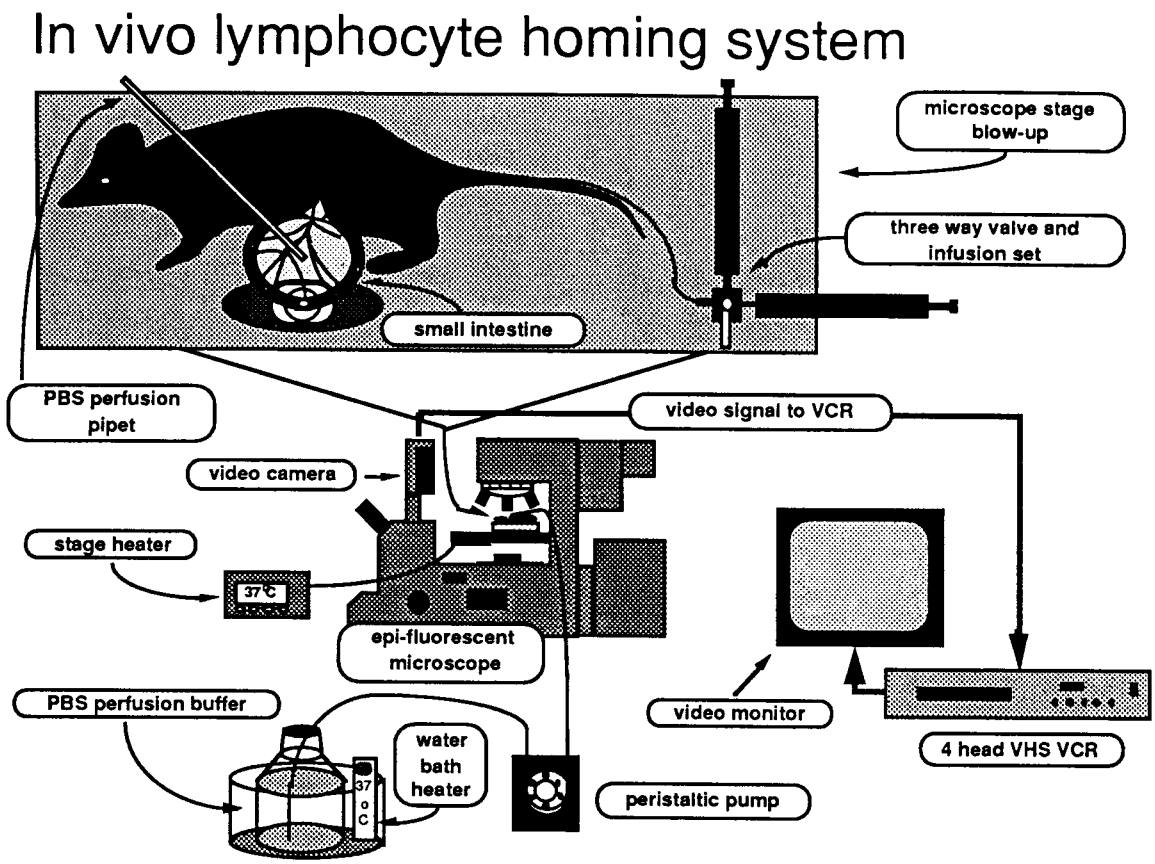


Figure 3. The in vivo video-microscopy recording system was developed to analyze leukocyte-endothelial-cell interactions in the animal. This method makes detailed examination of blood-infused FITC-labeled lymphocyte interactions with high endothelial venules (HEVS) in exteriorized mouse Peyer's patches possible.

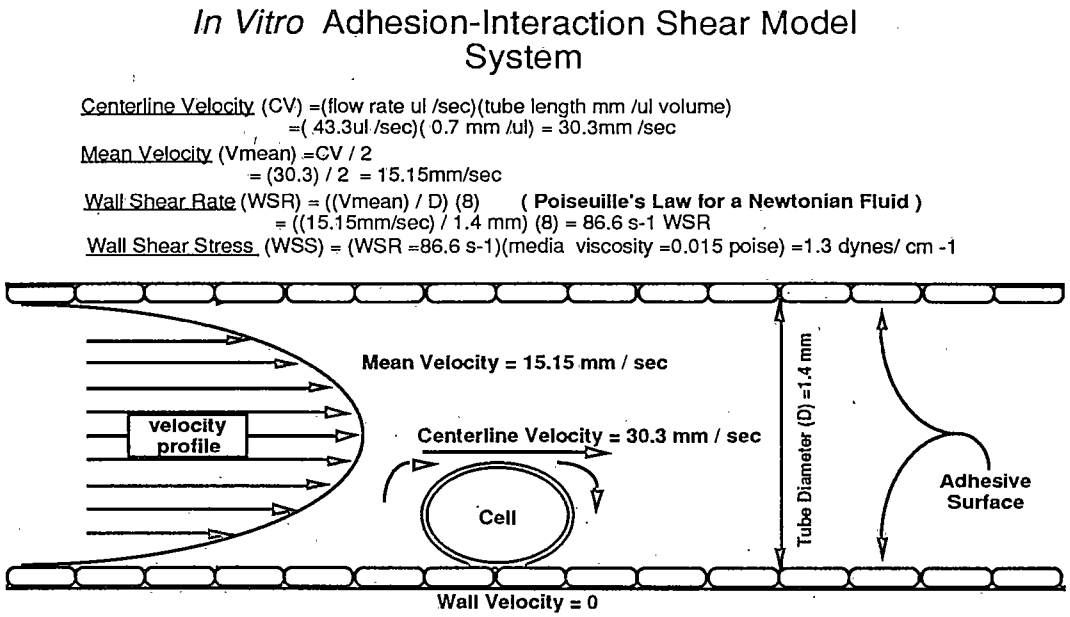
What are the "adhesokine" factors responsible for signaling lymphocyte strong adhesion to endothelium during inflammatory recruitment?

It is now generally assumed that IL-8 treatment of neutrophils promotes neutrophil strong adhesion under flow to endothelium by induction of CD11-CD18 activation [reviewed in Kelvin et al., (62); Shaw and

Adams, (63)]. In human skin, presentation of IL-8 by postcapillary venular endothelial cells has been demonstrated. IL-8 can also induce neutrophils to migrate along a gradient in vitro, suggesting it is likely to cause neutrophils to emigrate and migrate into inflamed tissues [Rot, (64)]. Other factors that possess similar properties and protein structures have been identified for neutrophils (MGSA) and monocytes (MCAF). This family of peptides has been termed "chemokines," with each member having proinflammatory activity, and belonging to one of two subfamilies: the "α" C-X-C or the "β" C-C. The C-X-C and C-C nomenclature refers to presence or absence of an intervening amino acid between the first two of four conserved cysteines in the peptide sequence. Chemokines act on leukocytes through the serpentine family of the seven transmembrane-spanning G-protein-coupled receptors and are involved in rapid signaling events. This receptor class is responsible for signal transduction exemplified by the rodopsin receptor in visual perception, where high speed signaling is an absolute requirement. The pertussis toxin blocking of integrin-mediated lymphocyte strong adhesion, reported in Chapter 2 of this thesis, likely occurs through this class of coupled signaling pathway.

Chemokines have been identified that target lymphocytes, upregulating their static surface-binding capacities (reviewed in Miller and Krangel, (65)]. It has been hoped that the lymphocyte chemokines would exhibit the same rapid activation properties that IL-8 shows for neutrophils, inducing integrin activation to support slowing and tight adhesion during lymphocyte-endothelial cell rolling. The RANTES chemokine was the first shown to be active on T lymphocytes, stimulating chemotaxis and adhesion

[Oppenheim et al., (66); Schall, (67)]. Studies by Kelvin (62) and Shaw (63) have shown that chemokines will upregulate the adhesiveness of lymphocytes for isolated VCAM-1 or activated endothelium after 1-24 hr long preincubations in culture. Individual chemokines such as MIP-1α & β acting on CD8 and CD4 T cells respectively, appear to act most effectively on the memory T-cell populations. Vasoactive intestinal peptide (VIP) [Ottaway, (68)], a 28-amino acid peptide of the glucagon-secretin family, also interacts with a serpentine G-protein-coupled lymphocyte cell-surface receptor. VIP has the property of acting on unstimulated T cells, promoting increased binding to VCAM and ICAM in static assays. Based upon these observations of lymphocyte chemokine and VIP-induced adhesive activity, researchers have proposed that these small peptides may act to signal lymphocyte tight adhesion during rolling at sites of inflammation [Johnston et al., (69)]. Chapter 4 of this thesis reports data from an ongoing project for which a manuscript is currently in preparation. We examine the effect of chemokine treatment on lymphocytes in the in vitro loop HUVEC shear binding assay and plan to investigate the VIP effects in future studies. The effects of the chemokines have been examined in detail using the system described in Figure 4.



Reference: Perry, M. A. and Granger, D. N.. J. Clin. Invest. 87:1798-1804

Figure 4. Calculations for the typical in vitro shear system, shown in this figure, provide detailed information describing cell-cell/substrate interactions. These include rolling speed, rolling behavior, number of cells binding per unit area versus time, and the effects of modulators of these interactions allowing comparison of leukocyte subsets under a variety of experimental conditions. Either leukocytes and/or endothelial cells can be activated in this system using a spectrum of cytokines. Blocking of interactions by mAb, enzymatic treatment, or direct chemical inhibitors can be observed and accurately quantified.

Acute inflammation-driven neutrophil and lymphocyte localization and selective recruitment to inflammatory sites bear striking similarities and differences.

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Comparing the recruitment of lymphocytes and neutrophils to acute sites of inflammation suggests that both the myeloid and lymphoid subsets

appear to use a three-step process of recognition and binding to the endothelium at these sites [Shaw in (63)]. What is truly of interest is that these different cell types do not simultaneously compete for endothelial binding, but are recruited to the site of inflammation in a temporally distinct sequence. This was clearly demonstrated by Jutila et al. (70) where, in leukocyte recruitment to thioglycolate-inflammed peritoneum, they showed that the first cell population to be recruited was neutrophils, followed by monocytes, and finally lymphocytes. This work and the work of others provide a potentially clear explanation for this sequential regulation. That is, there is a temporally regulated expression of adhesion receptors and adhesokines on the endothelium specific for the leukocyte subset that is selected at any time point for recruitment. The temporal regulation of these molecules can be directly related, as will be discussed in Chapter 4, to the production of cytokines at the inflammatory site. Thus, an understanding of the nature of the molecular requirements for early neutrophil recruitment in Chapters 5 and 6 will provide a means of identifying those requirements that are unique for the recruitment of lymphocytes vs neutrophils to acute inflammatory sites.

The behavior of leukocytes in the microvascular circulation has been of interest to scientists for over a century [von Andrian et al., (3)]. The mechanisms and adhesion receptors regulating the recruitment process for leukocytes to endothelium were largely unknown until the 1980s. This knowledge has largely been gained through the use of mAbs selected to identify and block the function of the adhesive process. To the surprise of

many researchers, many unique adhesion-receptor pairs have been identified for leukocytes and endothelial cells. This is likely due to the versatility that has developed in host defense to select a specific leukocyte subpopulation to react at key times during the progression of an inflammatory episode. The endothelium during inflammation responds to pro-inflammatory cytokines, such as IL-1, IL-4, γ interferon, and TNF, as released by the inflamed tissue, each inducing differential expression of E-selectin, VCAM-1, and ICAM-1 and yet-to-be-defined receptors on the endothelial surface. These adhesion-supporting cell-surface glycoproteins provide the adhesive foothold needed to initiate rolling and subsequent leukocyte arrest, as I have previously described for the lymphocyte. If the correct receptors and counter ligands are present on the leukocyte and endothelium, the circulating cell, rolling slowly relative to the blood flow, will find accessible signaling molecules on the endothelium capable of altering the avidity of rolling adhesion. Gradients of these chemotactic/chemokine factors may be presented by endothelial surface proteoglycan molecules acting to induce strong adhesion of leukocytes. After stopping, these factors and/or others are likely required for subsequent transmigration [Tanaka et al., (71)].

A first critical observation by Kishimoto et al. (72) provided insight into the consequences of chemotactic factor action on neutrophil adhesion receptor regulation. They found that activation of the cells resulted in rapid cleavage of L-selectin form the surface and equally rapid upregulation of Mac-1 expression on the neutrophil surface. The consequences of this regulation clearly showed that if L-selectin were cleaved before firm

attachment to the endothelium, the neutrophil would return to the blood flow. However, if the upregulated Mac-1 had cemented the neutrophil to the blood-vessel wall, the loss of L-selectin might ease the process of transendothelial migration. This hypothesis was in fact confirmed by Jutila et al. (70) in vivo where neutrophils that had migrated into an inflammatory site lost their L-selectin and had greatly upregulated their expression of Mac-1.

As has been discussed, a number of adhesion receptors and their ligands, such as Mac-1 and ICAM-1, were identified for having adhesive function that could be blocked by mAbs under static assay conditions. In fact a leukocyte adhesion deficiency (LAD-1) was identified as a deficit in the expression of the Mac-1, LFA-1, p150,95 integrin glycoproteins. But, Arfors et al. (56) were the first to show that the ß2 integrin MAC-1, blocked by an anti-CD18 mAb, inhibited accumulation of neutrophils in a dynamic in vivo model. This work was elaborated on in a paper by Ley et al. (73) who first showed that L-selectin was responsible for mediating neutrophil rolling in the same in vivo rabbit mesenteric venule model used to describe CD18 function.

These in vivo observations set the stage for the first in vitro selectin rolling model which was presented by Lawrence and Springer (74). In their paper, where P-selectin was demonstrated to act as a ligand that supports neutrophil rolling, the authors also proposed that rolling interactions were a prerequisite for engagement of MAC-1 with ICAM-1. This paper was rapidly followed by the finding that E-selectin also supported neutrophil rolling under flow conditions Kishimoto et al., (75). Since P-selectin is rapidly

upregulated on endothelial cells and platelets, it was hypothesized that P-selectin would be rapidly downregulated after providing a rapid response mechanism for recruitment of neutrophils to an acute inflammatory site. E-selectin, requiring several hours for endothelial cell surface expression, would then support additional accumulation if the inflammatory site continued to mature. This may not hold true in vivo since the regulation of these molecules was extrapolated from in vitro data.

Kishimoto et al. (75) again presented new results demonstrating that L-selectin and E-selectin act in a common adhesion pathway which suggests these two selectins may be counter ligands in neutrophil rolling and recruitment. Picker et al. supported this observation and suggested it was also true for L-selectin and P-selectin. New ligands have been demonstrated for both E- and P-selectin on neutrophils [Levinovitz et al., (76); Moore et al., (77); Lenter et al., (78)] and the full characterization and identification of their role of participation in binding to endothelial cells is currently under way.

We have very recently published a paper in the Journal of Immunology confirming the participation of L- and E-selectin in human neutrophil binding to inflamed HUVECs. Chapter 5 of this thesis reports these findings in detail, describing the properties of a unique anti-human mAb EL-246 that binds and blocks the adhesive functions of both L- and E-selectin and has profound effects when used as a clinical therapeutic. This antibody possesses the biochemical characteristic of "jumping" to E-selectin from L-selectin when it is prebound by L-selectin on neutrophils and then co-incubated with E-selectin transfectants or stimulated HUVECs. Because of the EL-246 "jumping" property, the leukocyte can directly deliver this mAb

to the endothelium. Therefore, it may be possible to use smaller dosages than used for other anti-selectin mAbs to get the same adhesion blocking effect. This mAb binds and blocks L- and E-selectin from many species and, as we report, it is very effective in vivo, as seen in blocking bovine lymphocyte homing to mouse PLN. The wide effectiveness of this antibody may make it useful for treatment of animal inflammatory diseases, as well as for humans.

During the course of examining neutrophil-endothelial interactions with activated HUVEC, as reported in the November issue of The Journal of Experimental Medicine and Chapter 6 of this thesis, we observed a new previously unreported phenomenon of neutrophil rolling. These findings have provided evidence for a new theory describing the augmented recruitment of neutrophils to acute inflammatory sites which may be generalized to numerous leukocyte subsets in a variety of inflammatory settings.

Statement of the hypothesis of leukocyte recruitment to the vascular endothelium.

The global hypothesis to be tested is that leukocyte recruitment to the vascular endothelium is a rapid, multi-step process, regulated at each of three sequential events. This process can be accurately termed an "adhesion cascade." The intent of this thesis has been to define the "adhesion cascade" by 1) characterizing the active process of leukocyte-endothelial cell recruitment; 2) defining the individual roles of leukocyte-endothelial cell

adhesion receptors during recruitment; and 3) determining the means by which endothelial-leukocyte signaling can occur to induce leukocyte adhesion strengthening for arresting leukocytes from the blood flow.

First I have used direct observation of lymphocyte homing to Peyer's patch high endothelial venules in vivo as a means to formulate a molecular-based theory of lymphocyte homing. Second, by observing the rolling and attachment of human neutrophils and lymphocytes to the surface of activated HUVEC in an in vitro shear model, I have formed the basis for my theory of acute inflammatory site recruitment of leukocytes.

References

1. Tanaka, Y., D. H. Adams, S. Shaw. 1993. Proteoglycan on endothelial cells present adhesion-inducing cytokines to leukocytes. Immunol. *Today. 14:111.*

2. van Kooyk, Y., E. van de Wiel-van Kemenade, P. Weder, R. J. F. Huijbens, C. G. Figdor. 1993. Lymphocyte function-associated antigen 1 dominates very late antigen 4 in binding of activated T cells to endothelium. *J. Exp. Med. 177:185.*

3. von Adrian, U. H., J. D. Chambers, L. M. McEvoy, R. F. Bargatze, K. E. Arfors, E. C. Butcher. 1991. Two step model of leukocyte-endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte beta 2 integrins *in vivo. Proc. Natl. Acad. Sci. USA 88. 7538: 7542.*

4. Butcher, E. C.1991. Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell. 67:1033.*

5. McEver, R. P. 1992. Leukocyte-endothelial interactions. *Curr. Opin. Cell. Biol. 4:840.*

6. Lawrence, M. B., T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flows rates: Distinction from and prerequisite for adhesion through integrins. *Cell. 65:859.*

7. Shimizu, Y., W. Newman, Y. Tanaka, S. Shaw. 1992. Lymphocyte interactions with endothelial cells. Immunol. *Today. 13:106.*

8. Kishimoto, T. K., M. A. Jutila, E. L. Berg, et al. 1989. Neutrophil mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science. 245:1238-1241.*

9. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. Lond. Ser. B. 159:257.*

10. Reichert, R. A., W. M. Gallatin, I. L. Weissman, and E. C. Butcher. 1983. Germinal center B cells lack homing receptors necessary for normal lymphocyte recirculation. *J. Exp. Med. 157:813.*

11. Butcher, E., R. Scollay, and I. Weissman. 1979. Lymphocyte adherence to high endothelial venules: Characterization of a modified *in vitro* assay, and examination of the binding of syngeneic and allogeneic lymphocyte populations. *J. Immunol. 123:1996.*

12. Stevens, S. K., I. L. Weissman, and E. C. Butcher. 1982. Differences in the migration of B and T lymphocytes: Organ-selective localization *in vivo* and the role of lymphocyte-endothelial cell recognition. *J. I mmunol. 128:844.*

13. Butcher, E., R. Scollay, and I. Weissman. 1980. Organ specificity of lymphocyte interation with organ-specific determinants on high endothelial venules. *Eur. J. Immunol. 10:556.*

14. Jalkanen, S., A. Steere, R. Fox, and E. C. Butcher. 1986c. A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. *Science. 233:556-558.*

15. Stamper, H. B., Jr. and J. J. Woodruff. 1976. Lymphocyte homing into lymph nodes: In vitro demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. *J. Exp. Med. 144:828.*

16. Bargatze, R. F., N. W. Wu, I. L. Weissman, and E. C. Butcher. 1987. High endothelial venule binding as a predictor of the dissemination of passaged murine lymphonas. *J. Exp. Med. 166:1125.*

17. Gallatin, W. M., I. L. Weissman, and E. C. Butcher. 1983. A cell surface molecule involved in organ-specific homing of lymphocytes. *Nature. 304:30.*

18. Rasmussen, R. A., Y. -H. Chin, J. J. Woodruff, and T. G. Easton. 1985. Lymphocyte recognition of lymph node high endothelium. VII. Cell surface proteins involved in adhesion defined by monoclonal anti-$HEBF_{LN}$ (A.11) antibody. *J. Immunol. 135:19.*

19. Lewinsohn, D. M., R. F. Bargatze, and E. C. Butcher. 1987. Leukocyte-endothelial cell recognition: Evidence of a common molecular mechanism shared by neutrophils, lymphocytes and other leukocytes. *J. Immunol. 138:4313.*

20. Stoolman, Lloyd M. 1989. Adhesion Molecules Controlling Lymphocyte Migration. *Cell. 56:907-910.*

21. Yednock, T. A., E. C. Butcher, L. M. Stoolman, and S. D. Rosen. 1987. Receptors involved in lymphocyte homing: Relationship between a carbohydrate-binding receptor and the MEL-14 antigen. *J. Cell. Biol. 104:725.*

22. Siegelman, M. H., M. van de Rijn, and I. L. Weissman. 1989. Mouse lymph node homing receptor cDNA clone encodes a glycoprotein revealing tandem interaction domains. *Science. 243:1165.*

23. Lasky, L. A., M. S. Singer, T. A. Yednock, D. Dowbenko, C. Fennie, H. Rodriguez, T. Nguyen, S. Stachel, S. D. Rosen. 1989. Cloning of a lymphocyte homing receptor reveals a lectin domain. *Cell. 56:1045.*

24. Watson, S. R., Y. Imai, C. Fennie, J. Geoffrey, M. Singer, S. D. Rosen, L. A. Lasky. 1991. The complement binding-like domains of the murine homing receptor facilitate lectin activity. *J. Cell Biol. 115:235.*

25. Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr., and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory. proteins and lectins. *Science. 243:1160.*

26. Johnston, G. I., R. G. Cook, and R. P. McEver. 1989. Cloning of GMP-140, a granule membrane protein of platelets and endothelium: Sequence similarity to proteins involved in cell adhesion and inflamamtion. *Cell. 56:1033.*

27. Bargatze, R. F., P. R. Streeter, and E. C. Butcher. 1990. Expression of low levels of peripheral lymph node-associated vascular addressin in mucosal lymphoid tissues: Possible relevance to the dissemination of passaged AKR lymphomas *J. Cell Biochem. 42:219-227.*

28. Hamann, A., D. Jablonski-Westrich, P. Jonas, and H. Thiele. 1991. *G. Eur. J. Immun. 21:2925-2929.*

29. Streeter, P. R., B. T. Rouse, and E. C. Butcher. 1988*b*. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol. 107:1853-1862.*

30. Berg, E. L., L. A. Goldstein, M. A. Jutila, M. Nakache, L. J. Picker, P. R. Streeter, N. W. Wu, D. Zhou, and E. C. Butcher. 1989. Homing receptors and vascular addressins: Cell adhesion molecules that direct

lymphocyte traffic. *Immunol. Rev. 108:5-18.*

31. Berg, E. L. , M. K. Robinson, R. A. Warnock, and E. C. Butcher. 1991. The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J. Cell Biol. 114:343-349.*

32. Michie, S. A., P. R. Streeter, P. A. Bolt, E. C. Butcher, and L. J. Picker. 1993. The human peripheral lymph node vascular addressin. *Am. J. Path. 143:1688.*

33. Hanninen, A., Taylor, C., Streeter, P. R., Stark, L. S., Sarte, J. M., Shizuru, J. A., Simell, O., and S. A. Michie. 1994. Vascular addressins are induced on islet vessels during insulitis in NOD mice and are involved in lymphoid cell binding to islet endothelium. *J. Clin. Invest. in press.*

34. Berg, E. L., L. J. Picker, Robinson, M. K., Streeter, P. R. and E. C. Butcher. 1991. in *Cellular and Molecular Mechanisms of Inflamation. Vol. 2.* (eds Cochrane, C. G. and M. A. Gimbrone). Academic. New York. pgs. 111-129.

35. Berg, E. L., L. M. McEvoy, C. Berlin, R. F. Bargatze, and E. C. Butcher. 1993. L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature. 366:695.*

36. Chin, Y. -H., R. A. Rasmussen, J. J. Woodruff, and T. G. Easton. 1986. A monoclonal anti-HEBFpp antibody with specificity of lymphocyte surface modecules mediating adhesion to Peyer's patch high endothelium of the rat. *J. Immunol. 135:188.*

37. Jalkanen, S., R. F. Bargatze, J. de los Toyos, and E. C. Butcher. 1987. Lymphocyte recognition of high endothelium: Antibodies to distinct epitopes of an 85-95 kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal or synovial endothelial cells. *J. Cell. Biol. 105:983.*

38. Goldstein, L. A., D. F. H. Zhou, L. J. Picker, C. N. Minty, R. F. Bargatze, J. F. Ding, and E. C. Butcher. 1989. *Cell. 56:1063.*

39. Schmitz, M., D. Nunez, and E. C. Butcher. 1988. Selective recognition of mucosal lymphoid high endothelium by gut intraepithelial leukocytes. *Gastroenterology. 94: 576-581.*

40. Holtzmann, B., B. W. McIntyre, and I. L. Weissman. 1989. *Cell. 56:37-46.*

41. Issekutz, T. B. 1991. Inhibition of *in vivo* lymphocyte migration to inflammation and homing to lymphoid tissues by the TA-2 monoclonal antibody. A likely role for VLA-4 *in vivo. J. Immunol. 147:4178-4184.*