1. Introduction

1.1. Cell adhesion: the basis of multicellular life

The capability of cells to adhere to each other and to the secreted macromolecules that constitute the extracellular matrix (ECM) is the basic requirement for the existence of multicellular organisms. Adhesive interactions enable cells to form cooperative assemblies called tissues, which again associate to compose larger functional units, the organs.

The most eminent role for adhesion molecules is found in the sheet-like cell layers that constitute epithelial and endothelial tissues. Epithelial cells form the skin and cover all surfaces inside the body that are in contact with the exterior, like the intestine, glands and the respiratory tract. The single cell layer of the endothelium coats the luminal surface of vessels in blood and lymph circulation. Cells in these layers are held together and attached to the underlying extracellular matrix by a variety of cell adhesion molecules that are organised into large complexes. Tight junctions at the lateral faces of neighbouring cells prevent uncontrolled leakage of macromolecules into the tissue and maintain the polarity of the cells characterised by the distinct distribution of surface molecules to the apical or basolateral surface. Stability of the cell layer is created by adherence junctions and desmosomes between cells, complex structures that link the adhesion molecules to the actin and intermediate filaments of the cytoskeleton to provide mechanical strength. Similar organised structures exist at the basal side of the cells, where they firmly attach the cell to the basal lamina, a thin layer of extracellular matrix molecules underlying the epithelium (Hynes, 1999). Although the cellular interactions in epithelial sheets may appear static, they are subject to constant modulation. Being more than a tight barrier between different environments, these tissues have to transiently loosen contacts to allow controlled passage of macromolecules and even whole cells.

Still more plastic cellular interactions are required during the development and regeneration of complex organisms. Cells have to abandon existing associations, leave their original environment and use transient adhesion interactions to migrate to new locations, were they again establish firm connections with neighbouring cells to initiate formation of tissues and organs. To meet the requirement for both stability and plasticity, adhesion molecules must be able to form strong associations to maintain tissue architecture against forces exerted by the environment, but on the other hand they have to respond to the needs of the organism for modulation of existing structures (Buckley *et al.*, 1998). This flexibility is achieved by alteration of the binding properties of individual adhesion molecules as well as by tight regulation of temporal and spatial expression patterns of the molecules on the cell surface.

1.2. Cell adhesion in the immune system

In contrast to many other cell types, the white blood cells of the immune system do not form large multicellular aggregates, but rather exist as single cells

that patrol the blood and lymphoid system. Nevertheless, cell adhesion events are required for these cells to communicate with each other and to cooperate in the defence against invading pathogens. Adhesion molecules on immune cells mediate interactions of different leukocyte populations, e.g. during activation of lymphocytes by T-helper and antigen-presenting cells, and adhesion of leukocytes to vessel walls at the sites of extravasation.

Extravasation is an essential step in the immune response. Leukocytes travel through the body within the blood stream and lymphoid system most of their life, but they have to leave the circulation and enter various tissues to exert their immunological function. Different blood cell populations use different routes for entrance into extravascular sites. Monocytes and granulocytes, responsible for the unspecific immune response, migrate to sites of tissue damage and inflammation to eliminate bacterial invaders and other pathogens (Jutila, 1992). Lymphocytes, which mediate the antigen-specific immune reaction, are also recruited to sites of inflammation, and additionally recirculate to lymphatic tissues where they are likely to encounter their specific antigens, a process described as lymphocyte homing (Butcher and Picker, 1996). Mature naïve T- and B-cells, which have not yet been activated by antigen, enter secondary lymphatic tissues like peripheral lymph nodes, Peyer's patches associated with intestine, or tonsils. Antigens from neighbouring body areas are collected in these organs, where they are processed by resident antigen presenting cells and displayed together with co-stimulatory signals for activation of lymphocytes with appropriate specificity. A subset of activated lymphocytes differentiate into memory cells with an extended life span. These cells show a recirculation pattern different from that of naïve lymphocytes, preferentially homing to lymphatic or non-lymphatic sites where they have first encountered their antigen.

1.3. The leukocyte adhesion cascade

Leukocyte extravasation mainly takes place in postcapillary venules at inflammatory sites or at specialised sections of vessels in lymphatic tissue, the high endothelial venules (HEV) (Girard and Springer, 1995). The general adhesion mechanisms involved in leukocyte recruitment during inflammation and lymphocyte recirculation are very similar. Extravasation at specific sites is achieved by the coordinated employment of a number of adhesion molecules on both the leukocyte and the endothelial wall (Springer, 1994). The interactions between these molecules constitute an adhesion cascade (see also Fig. 1) that can be divided into several distinct, although overlapping steps:

- initial capture of the leukocyte from the blood stream and slow rolling along the vessel wall by means of weak and transient interactions of adhesion molecules
- activation of leukocytes by mediators displayed on the endothelial surface that leads to increased adhesiveness
- tight binding to the endothelial cell layer assisted by spreading of the lymphocytes on the surface
- migration towards endothelial cell boundaries and transmigration (diapedesis) into the subendothelial space
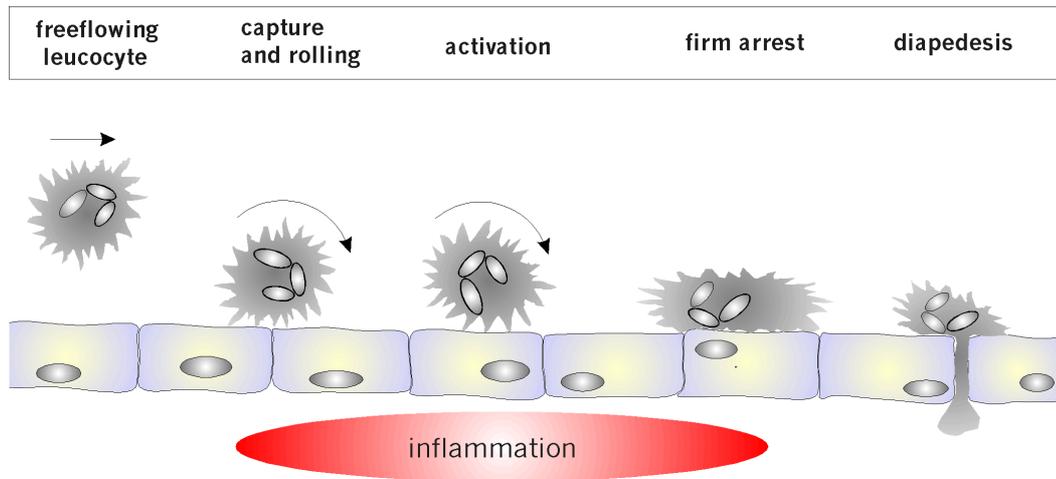
Fig. 1: The leukocyte adhesion cascade.
At sites of inflammation and in lymph node HEV leukocyte extravasation occurs in several consecutive steps. Cells from the blood stream make initial contact with the vessel wall, resulting in slow rolling on the endothelial cell layer. Activation of rolling cells induces tight adhesion and flattening of the leukocyte, followed by migration to endothelial cell boundaries and diapesesis into the underlying tissue.

Specificity in the extravasation of different leukocyte subsets is achieved by the combination of adhesion molecules present on the endothelial cells and the blood cell. Thus, leukocytes can only extravasate at sites where they encounter an set of adhesion molecules exactly matching the ones expressed on their surface. This allows the precise recruitment of specific leukocyte populations to sites in the body where their immunological functions is required.

Several families of adhesion molecules participate in the extravasation cascade (Fig. 2, Tab. 1). Their properties are described briefly before their particular roles in the different steps of the adhesion cascade are considered in detail.

1.3.1 Adhesion molecules involved in leukocyte extravasation

Selectins

The selectins are a family of monomeric transmembrane glycoproteins with carbohydrate-binding properties (Bevilacqua and Nelson, 1993; Tedder *et al*., 1995a). The selectin-family consists of three members named according to their expression pattern: E-selectin is expressed on activated vascular endothelium (Bevilacqua *et al*., 1987), P-selectin was first identified on platelets but is also found on activated endothelium (Hsu-Lin *et al*., 1984) and L-selectin is expressed exclusively on leukocytes (Gallatin *et al*., 1983). Whereas L-selectin expression is constitutive on granulocytes, monocytes, naïve lymphocytes and a subset of memory T-cells, the expression of vascular selectins is induced by proinflammatory mediators and signal derived from tissue damage. P-selectin is stored in secretory granules (Weibel-palade bodies in endothelial cells or α-granules in platelets) and mobilised to the cell surface within minutes following activation, where it has a very limited life span before it is internalised (McEver,

1993). E-selectin is transcriptionally regulated, with peak expression about 4-6 hours following induction (McEver, 1994; Tedder *et al*.,1995a).

All selectins have a homologous structure with a N-terminal C-type lectin domain which is essential for carbohydrate ligand binding, an epidermal growth factor (EGF) domain, a varying number of short consensus repeats (two in L-selectin, six in E-selectin and nine in P-selectin) common in complement binding proteins, a transmembrane domain and a short cytoplasmic domain.

Selectins establish Ca^{2+}- dependent interactions with carbohydrate groups related to the sialylated and fucosylated sialyl-LewisX tetrasaccharide (Nelson *et al*., 1995). For L-selectin binding, sulphation of sugar groups is also required. The carbohydrate moieties acting as selectin ligands are displayed in the context of glycosylated proteins, in most cases known to date as O-linked glycans on mucin-like molecules (Varki, 1997).
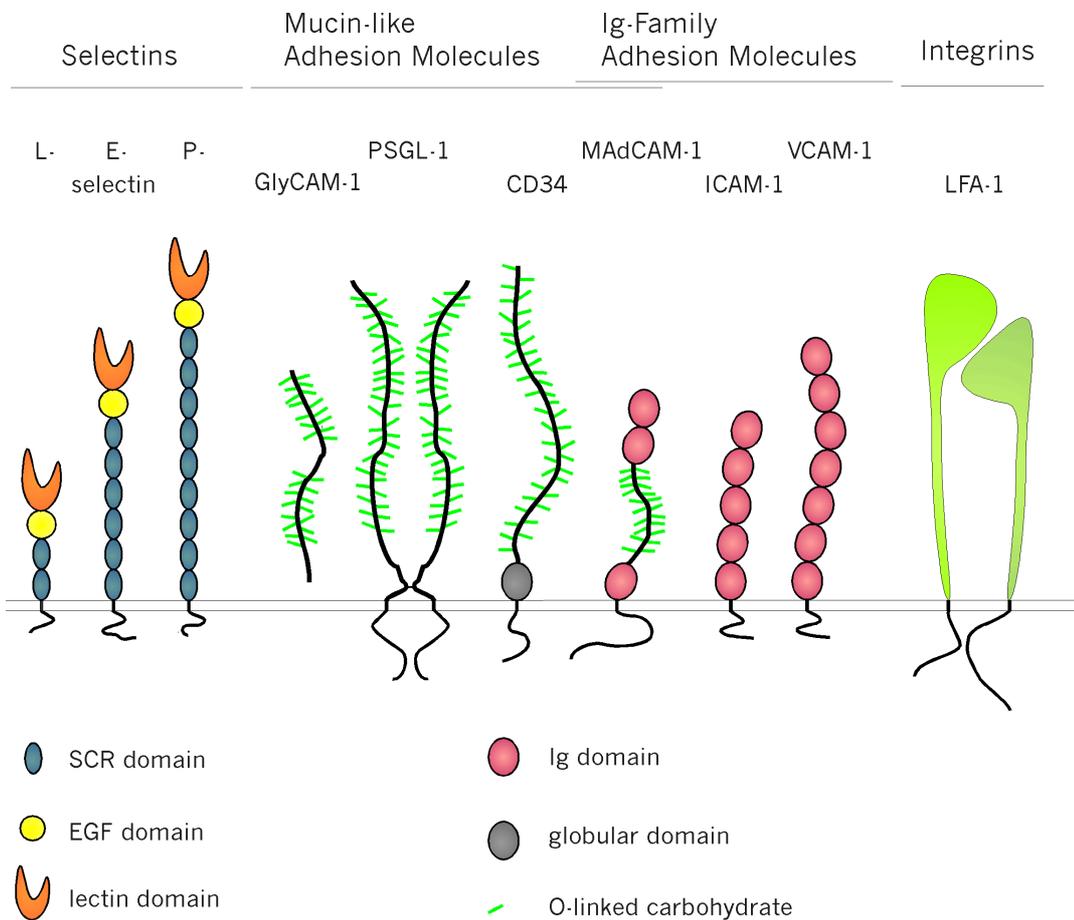
Fig. 2: Adhesion molecules involved in leukocyte extravasation.
Schematic representation of the domain structure of members of the different cell adhesion molecule (CAM) families that have a role in leukocyte extravasation.

Leukocyte adhesion molecule	Expression pattern	Endothelial binding partner	Expression pattern	Function
VLA-4 ($\alpha_4\beta_1$)	monocytes, lymphocytes	VCAM-1	activated endothelium	rolling, tight adhesion and migration inflammatory response
LFA-1 ($\alpha_L\beta_2$)	all leukocytes	ICAM-1, ICAM-2	activated endothelium, HEV	firm adhesion and transmigration inflammatory response and lymphocyte homing
Mac-1 ($\alpha_M\beta_2$)	granulocytes, monocytes	ICAM-1	activated endothelium, HEV	firm adhesion and transmigration inflammatory response
$\alpha_4\beta_7$	lymphocyte subpopulations	MAdCAM-1	mesenteric lymph nodes, Peyer's patch HEV	Rolling and tight adhesion homing of naïve lymphocytes to gut-associated lymphoid tissues (Peyer's patches and appendix), homing of memory lymphocytes to mucosal sites
		VCAM-1	activated endothelium	
ESL-1 (E-selectin ligand-1)	myeloid cells	E-selectin	activated endothelium	rolling inflammatory response
CLA (cutaneous lymphocyte -associated antigen)	skin homing memory T-cells	E-selectin	vascular endothelium close to skin lesions	rolling homing of memory T-cells to skin
L-selectin	granulocytes, monocytes, naïve lymphocytes, memory cell subsets	?	activated endothelium	rolling inflammatory response
		MAdCAM-1	mesenteric lymph nodes, Peyer's patches	rolling tissue naïve lymphocyte homing to mucosal lymphoid
		GlyCAM-1 CD34	HEV in peripheral lymph nodes	rolling lymphocyte homing to secondary lymphoid tissue
PSGL-1	all leukocytes	P-selectin	activated endothelium, activated platelets	rolling inflammatory response
PECAM-1	all leukocytes	PECAM-1	constitutive on endothelial cell junctions	transmigration, crossing of basal lamina inflammatory response and lymphocyte homing

Tab. 1: Adhesion molecules involved in leukocyte extravasation.

Integrins

Integrins are heterodimeric molecules with non-covalently associated alpha and beta chains. Several different alpha and beta chains are known to date, which are expressed in various, but distinct combinations on the surface of most cell types and are involved in many cell-cell and cell-matrix interactions (Newham and Humphries, 1996). Both integrin subunits are necessary for surface expression and ligand binding.

While most integrins on non-immune cells bind to molecules of the extracellular matrix like collagen and fibronectin, leukocyte-associated integrins mediate cell-cell interactions as well as ECM attachment (Hynes, 1992). Integrins are grouped according to their beta-chains. Several classes of integrins take part in the leukocyte extravasation process (Springer, 1994):

- **β_2-integrins**
  This class of integrins is found exclusively on leukocytes. The β_2 chain (CD18) associates with several alpha chains, the best characterised combinations being $\alpha_L\beta_2$ (lymphocyte function associated-1, LFA-1) on lymphocytes, granulocytes and monocytes and $\alpha_M\beta_2$ (Mac-1), which is expressed on granulocytes and monocytes

- **β_1-integrins**
  β_1-integrins constitute a large class mainly involved in ECM-binding, but $\alpha_4\beta_1$ (very late antigen-4, VLA-4) found on lymphocytes and monocytes also mediates adhesion to the vessel wall by binding to cellular surface molecules.

- **β_7-integrins**
  The β_7 chain also associates with α_4 to effect interaction of a subset of lymphocytes to endothelial cells.

The leukocyte integrins are constitutively expressed on the surface of different leukocyte subsets, but, best characterised in the case of β_2-integrins, require activation by proinflammatory mediators to exert their adhesive function (Hynes, 1992). All integrins involved in leukocyte extravasation bind to endothelial surface receptors belonging to the immunoglobulin superfamily.

Immunoglobulin (Ig) superfamily

This superfamily (SF) of cell surface proteins comprises a large number of diverse members, all containing one or more Ig-like domains as typical of antibody molecules (Edelman and Crossin, 1991). The Ig-like domain is characterised by two conserved cysteine residues positioned 55 to 75 amino acids apart, which form a disulfide bond stabilising the domain structure. Ig cell adhesion molecules (Ig-CAMs) have important roles in embryonic development, formation of the nervous system and in immune and inflammatory responses, where they are engaged in homophilic and heterophilic interactions (Petruzzelli *et al.*, 1999).

In the immune system, Ig-SF members, which are constitutively or inducibely expressed on endothelium, mainly serve as counter receptors for leukocyte integrins in several steps of extravasation (Carlos and Harlan, 1994;

Springer, 1995). The intercellular adhesion molecules (ICAMs) on endothelial cells are involved in interaction with the β_2-integrins LFA-1 (ICAM-1,-2) and Mac-1 (ICAM-1). The vascular cell adhesion molecule-1 (VCAM-1) binds to VLA-4 and $\alpha_4\beta_7$ integrin (Elices *et al*., 1990; Berlin *et al*., 1995). The mucosal addressin cell adhesion molecule-1 (MAdCAM-1) uses a Ig-domain to bind to $\alpha_4\beta_7$ integrin, but also possesses a mucin-like domain that mediates binding to L-selectin (Berg *et al*., 1993; Berlin *et al*., 1993; Briskin *et al*., 1993). Platelet-endothelial cell adhesion molecule-1 (PECAM-1), which is found on leukocytes and endothelial cells is involved in transmigration by establishing both homophilic and heterophilic interactions.

Mucin-like adhesion molecules

Mucin-like molecules are characterised by a rigid, extended structure. The protein backbones contain serine-threonine-rich stretches which are heavily O-glycosylated. The sialomucins CD34, GlyCAM-1, MAdCAM and PSGL-1 use mucin-like domains to display carbohydrate moieties important for the interaction with the lectin binding domain of L-selectin (Rosen, 1999).

1.3.2 Function of adhesion molecules in different steps of leukocyte extravasation

Capture and rolling

The predominant adhesion molecules mediating the initial capture and rolling of leukocytes along the vessel wall in both inflammatory responses and lymphocyte homing are the selectins (Ley, 1996; Springer, 1994; Carlos and Harlan, 1994). The low-affinity interactions of these proteins with their carbohydrate ligands are well suited for capture of the flowing lymphocyte from the blood stream and the initiation of slow rolling by repeated making and breaking of bonds.

During inflammation, proinflammatory cytokines like TNFα and IL-1 induce expression of the vascular selectins, E- and P-selectin, as well as endothelial ligands for the leukocytic L-selectin in capillaries close to the site of tissue damage. *In vitro* assays have shown that all three selectins can mediate leukocyte rolling on cytokine activated endothelium, although the contribution of the individual molecules to leukocyte attachment at different immunological sites in vivo is not completely understood. P-selectin supports leukocyte rolling by binding to the P-selectin glycoprotein ligand-1 (PSGL-1) which is present as a homodimer on all lymphocytes, monocytes and neutrophils. (Sako *et al*., 1993; Moore *et al*., 1995). E-selectin binds to E-selectin ligand-1 (ESL-1) on myeloid cells and cutaneous lymphocyte-associated antigen (CLA) on skin-homing lymphocytes (Berg *et al*., 1991; Levinovitz, *et al*., 1993). The L-selectin ligands on vascular endothelium in inflammatory responses have not yet been characterised on the molecular level, although L-selectin-mediated adhesion clearly has an important role in this process (Hallmann *et al*., 1991; Spertini *et al*., 1991c). This was shown by use of antibodies directed against L-selectin that inhibit neutrophil adhesion to cytokine-activated endothelial cells (Kishimoto *et al*., 1991) and a soluble L-selectin-IgG chimera which blocks neutrophil

accumulation at extravascular sites (Watson *et al.*, 1991). *In vivo,* antibodies against both L-selectin (Ley *et al.*, 1991; von Andrian *et al.*, 1991) and P-selectin (Dore *et al.*, 1993) also significantly inhibit leukocyte rolling under various conditions. Intravital microscopy to analyse leukocyte rolling in vessels, antibody blocking studies and mouse knock-out models were used to dissect the partially overlapping but distinct functions of these selectins. Mice deficient in P-selectin show severely impaired rolling at sites of tissue trauma early after injury, whereas L-selectin knockout mice initially display normal rolling, which is almost abolished one hour after induction of trauma (Ley *et al.*, 1995). Similar results were observed for leukocyte recruitment in a peritonitis model (Tedder *et al.*, 1995b). Thus, a temporal pattern for selectin function seems to exist, with P-selectin responsible for the rolling observed very shortly after tissue damage and L-selectin acting as the main rolling receptor at later time points. This corresponds well with the rapid mobilisation of P-selectin to the cell surface after endothelial activation. In addition, the analysis of leukocyte subsets recruited in different inflammatory models in the selectin knockout mice demonstrated functional differences for the selectins in recruitment of particular cell populations (Tedder *et al.*, 1995b; Johnson *et al.*, 1995).

In models where both P- and L-selectin were present at the same time, a synergistic function in sequential steps of the adhesion process was observed. In several experiments where rolling had been previously shown to be strongly P-selectin dependent, it was also significantly inhibited by L-selectin blocking antibodies (Ley *et al.*, 1995). In *in vitro* experiments, leukocyte rolling which was exclusively dependent on L-selectin occurred at a higher velocity than rolling mediated by vascular selectins (Ley *et al.*, 1993 and 1995). This indicates that L-selectin is responsible for establishing the very first contact of the leukocyte with endothelium, described as leukocyte 'capture', which precedes the initiation of a stable rolling movement (Ley and Tedder, 1995). L-selectin is perfectly suited for this task, since it is concentrated on the tips of the leukocyte microvilli protruding from the cell surface, and this localisation has been shown to be a prerequisite for efficient attachment (von Andrian *et al.*, 1995). For the subsequent slow rolling along the vessel wall the vascular selectins are required.

Inhibition of E-selectin function in various systems has only limited effects on leukocyte rolling and recruitment. E-selectin deficient mice exhibit almost normal inflammatory responses. In these mice however, P-selectin antibodies have a more severe effect on the inflammatory response than in wild type mice, showing that the functions of E- and P-selectin are partially redundant (Labow, 1994). E-selectin has a special role in recruiting memory T-cells to skin by binding to the cutanous lymphocyte antigen (CLA) expressed on this lymphocyte population (Picker *et al.*, 1990 and 1991)

L-selectin is the major rolling receptor for homing of naïve T-lymphocytes to peripheral lymph nodes, where vascular selectins are not present. This was demonstrated in L-selectin knockout-mice, which display severe defects in this homing pathway. Mice lacking L-selectin show a 90% reduction in the size and cellularity of peripheral lymph nodes due to reduced influx of lymphocytes via HEV (Arbones *et al.*, 1994). *In vivo* administration of blocking L-selectin antibodies also completely blocks migration of lymphocytes to lymph nodes (Hou *et al.*, 1995). In contrast to inflammatory responses, homing ligands for L-selectin

are constitutively expressed by specialised endothelial cells in the high endothelial venules of peripheral lymph nodes. HEV ligands for L-selectin have been characterised at the molecular level. CD34 and GlyCAM-1 display carbohydrate moieties required for the interaction with the L-selectin lectin domain (Butcher and Picker, 1996). GlyCAM-1 lacks a transmembrane domain and is secreted by high endothelial cells (HEC). Its physiological function is still controversial. It has been proposed to function as a soluble signalling molecule contributing to cellular activation or to act as a anti-adhesive regulator by binding to L-selectin in a soluble form (Hoke *et al.*, 1995; Hwang *et al.*, 1996). CD34, which is expressed on many cell types in the human body, requires HEV-specific glycosylation to serve as a L-selectin binding partner (Baumheter *et al.*, 1993). A third L-selectin ligand from HEV, sgp200, has not yet been further characterised, but might play an important role for lymphocyte homing, since it is strongly upregulated in CD34 and GlyCAM knock-out mice, which show no severe defect in homing (Kansas, 1996).

L-selectin also binds to the mucin-like domain of MAdCAM expressed on mucosal HEV, initiating leukocyte homing to Peyer's patches (Berg *et al.*, 1993).

Additional to selectins, the α_4-integrins VLA-4 and $\alpha_4\beta_7$ integrin can support rolling of leukocytes in some cases, although integrin-mediated rolling is less efficient and limited to low shear rates. Lymphocytes are able to use VLA–4 to roll on VCAM-1 (Alon *et al.*, 1995) and $\alpha_4\beta_7$ to roll on MAdCAM (Berlin *et al.*, 1993) in flow chamber systems. $\alpha_4\beta_7$ is mainly expressed on lymphocytes (but not on neutrophils) and has been shown to be involved in the homing of intraepithelial lymphocytes to gut mucosa (Cepek *et al.*, 1993). Also, naïve lymphocytes homing into Peyer's patches use L-selectin for initial capture but require $\alpha_4\beta_7$-integrin for sustained rolling (Bargatze *et al.*, 1995). Memory lymphocytes which show low L-selectin expression may use $\alpha_4\beta_7$ as their major rolling receptor. Like L-selectin, $\alpha_4\beta_7$ is located on the tips of microvilli and binds to the Ig-domain of the mucosal vascular addressin MadCAM-1 (Berlin, 1995).

Activation

The slow progress of the leukocyte in close proximity to the endothelial cell layer during rolling allows contact with chemoattractants and chemokines produced by endothelial cells. These factors may be secreted or surface-bound and cause activation of the rolling leukocyte, thereby promoting firm adhesion to the vessel wall. This is achieved mainly by functional upregulation of integrins (described in the next section). Several activating signals have been described for neutrophil migration to inflammatory sites. They include platelet activating factor (PAF), IL-8 and leukotriene B4 (Zimmermann *et al.*, 1990; Dunon *et al.*, 1996). Il-8 plays a essential role in neutrophil activation, since Il-8 receptor deficient mice do not recruit neutrophils efficiently to inflamed tissues (Cacalano *et al.*, 1994).

Leukocyte

Endothelial cell

Step 1: Capture and rolling
Selectins and sialomucins

Step 2: Activation
Chemokines and
serpentine receptors

Step 3: Tight adhesion
Integrins and
Ig-family members
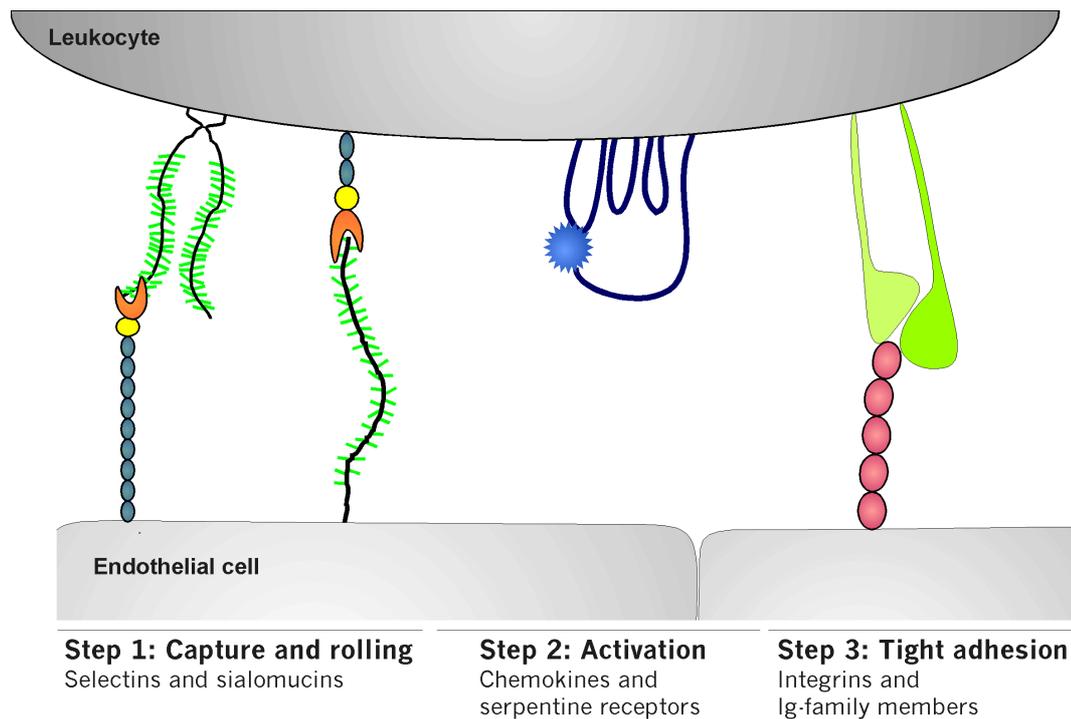
Fig. 3: Cell adhesion molecule families in different stages of the leukocyte extravasation cascade.
The steps of the adhesion cascade preceding emigration of leukocytes from vessels are typically mediated by members of distinct CAM-families (see also Fig. 2).

The activating chemoattractants and chemokines are produced by the endothelial cells that receive proinflammatory signals like TNFα and IL-1 from the underlying tissue. Many of these factors are retained close to their origin by binding to the surface of the endothelial cells, avoiding dilution into the bloodstream. Most chemokines consist of two distinct domains, one involved in binding to proteoglycans on the endothelial surface for presentation, while the other domain binds to serpentine receptors on the leukocyte (Dunon *et al*., 1996). These G-protein-coupled seven-span-transmembrane receptors initiate signalling events which result in triggering of intracellular signal cascades eventually increasing the adhesiveness of β_2-integrins ('inside-out' signalling). Blocking of the G-protein signalling pathway by pertussis toxin severely inhibits the integrin-dependent firm adhesion to endothelium (Larson and Springer, 1990).

There is evidence that, additional to activation by secreted factors, ligation of adhesion receptors during initial attachment to the vessel walls produces stimulatory signals. Crosslinking of L-selectin has been shown to result in partial activation of β_1- and β_2-integrins on neutrophils and lymphocytes (Simon *et al*., 1995; Sikorski *et al*., 1996). Binding of neutrophils to E-selectin also produced an activating response (Lo *et al*., 1991). Ligation of β_2-integrins themselves induced an oxidative burst and increased Ca^{2+} levels (Berton *et al*., 1992; Walzog *et al*., 1994).

The factors involved in induction of arrest during lymphocyte homing are less well characterised, although such a step is clearly required in this process.

Some recently characterised chemokines, including SDF-1, ELC and SLC, are constitutively expressed in lymphoid tissue and might play a role in lymphocyte activation (Baggiolini, 1998; Campbell *et al.*, 1998).

Firm adhesion and arrest

Integrins in their active state mediate firm adhesion to the vessel wall by binding to Ig-CAM ligands, followed by spreading of the cells and migration along chemotactic gradients through the endothelial cell layer and into the tissue.

Arrest of rolling cells is mainly promoted by β_2-integrins that are functionally upregulated by inflammatory signals. Activation is thought to involve conformational changes in the heterodimers leading to higher affinity for vascular ligands as well as clustering and rearrangement of the cytoskeleton (Hibbs *et al.*, 1991). Although Mac-1 expression on the cell surface is increased by mobilisation from intracellular storage granules, this quantitative upregulation seems not to be necessary for tight adhesion (Vedder and Harlan, 1988). LFA-1 and Mac-1 mediate arrest by binding to their Ig-CAM ligands on the endothelial cells. ICAM-1 is only weakly expressed on unactivated endothelium and is induced by proinflammatory stimuli. It serves as a ligand for both β_2-integrins. ICAM-2 is constitutively expressed, not upregulated during inflammation and recognised only by LFA-1 (Dustin *et al.*, 1989). Tight adhesion also involves binding of α_4 integrins to VCAM-1, that shows an increased expression in cytokine activated endothelium (Elices *et al.*, 1990; Schwartz *et al.*, 1990).

Adhesive interactions are enhanced by morphological changes of the cell. Leukocytes that are tightly bound to endothelial cells adopt a flattened appearance and spread along the vessel surface, increasing the area available for CAM interaction. Cellular responses are initiated by integrin-triggered outside-in-signalling after ligand binding that leads to activation of cytosolic kinases and modulation of gene expression (Humphries, 1996; Aplin *et al.*, 1998). These signals influence cell morphology, proliferation, differentiation (e.g. maturation of monocytes into macrophages) and migration.

Transmigration

Once arrested on the vessel wall, blood cells leave the lumen of the vessel and enter the underlying tissue. Diapedesis occurs mainly at junctions between endothelial cells, although a different, transcellular, route has been reported for the emigration of neutrophils from inflamed venules (Feng *et al.*, 1998; Johnson-Leger *et al.*, 2000).

Leukocytes are guided by chemotactic and haplotactic signals to migrate towards the endothelial cell-cell junctions. Transmigration involves the use of several adhesion receptors, mainly of the integrin- and Ig-CAM-families. The interaction of β_2-integrins with ICAM-1 is required for transmigration process (Furie *et al.*, 1991), and other integrins like VLA-4 and $\alpha_5\beta_1$ are also involved (Oppenheimer-Marks *et al.*, 1991). PECAM-1, which is concentrated at borders between apposing endothelial cells, mediates homophilic binding to PECAM-1 expressed on leukocytes and is essential for migration through the endothelial cell layer (Muller, 1993; Vaporciyan *et al.*, 1993). Leukocyte PECAM-1 is also necessary for crossing the endothelial basement membrane by heterophilic

interaction with ECM components (Wakelin *et al.*, 1996). The major adhesion molecule responsible for the integrity of endothelium is the vascular-endothelial (VE-) cadherin, which is located in the adherence junctions between the endothelial cells. Signalling events in endothelial cells triggered by attachment of leukocytes mediate loosening of cell adhesion by transient disruption of cadherin interactions, allowing passage of the migrating cells (Johnson-Leger *et al.*, 2000).

After diapedesis, migration inside the tissue towards the site of pathogen invasion is mainly mediated by β_1 and β_2-integrins (Bohnsack *et al.*, 1992).

1.3.3 Regulation of leukocyte trafficking by combinatorial diversity

The multitude of adhesion receptors, chemokines, other soluble activators and their respective receptors involved in leukocyte emigration, and the restricted expression of these molecules on certain types of leukocytes and endothelial cells produce a large array of combinatorial possibilities, which are used to selectively direct leukocyte subsets to specialised organs and to different sites of inflammation. The set of adhesion receptors and activating factors an endothelial layer expresses constitutes an "area code", with the result that only leukocyte subsets that express a complete set of matching receptors can extravasate at a certain site (Springer, 1994). The types of leukocytes emigrating and the kinetics of their recruitment has to be tightly regulated to ensure appropriate immune responses and to avoid overshooting reactions. The loss of adhesive interactions as well as undue stimulation of adhesion gives rise to a multitude of disease states (Tab. 2, Etzioni 1996; Petruzelli *et al.*, 1999).

Tab. 2: Diseases connected to adhesive dysregulation.

Disease	Nature of defect	Phenotype
Leukocyte adhesion deficiency-1 (LAD1)	decreased or absent expression of β_2-integrins due to defect in CD18	severe bacterial infections chronic gastrointestinal and pulmonary infections chronic neutrophilia (Anderson and Springer, 1987)
Leukocyte adhesion deficiency-2 (LAD2)	no expression of functional selectin ligands due to lack of fucosyl-transferase	recurrent infections neurological defects neutrophilia Growth retardation (Etzioni *et al*, 1993)
Arteriosclerosis	increased expression of endothelial adhesion molecules local activation of β_2-integrins	leukocyte accumulation endothelial damage due to reactive leukocyte metabolites (Ross and Vetvicka, 1993, Gibbons and Dzau, 1996)
Ischema-reperfusion injury	increased expression of endothelial adhesion molecules	leukocyte accumulation tissue damage (Thiagarajan *et al*, 1997)
Chronic inflammations (e.g. Crohn's disease, rheumatoid arthritis)	development of HEV-type vessels	constant lymphocyte infiltration (Girard and Springer, 1995)

The importance of selectins and β_2-integrins in leukocyte extravasation is demonstrated by two classes of leukocyte adhesion deficiency (LAD) syndromes in humans. They are characterised by recurrent and chronic infections due to impaired leukocyte recruitment. LAD1 is caused by a lack of expression of the CD18 (β_2) integrin subunit preventing arrest of blood cells on the endothelium. (Anderson and Springer, 1987). LAD2 results from a defect in fucosylation, which impairs expression of appropriately glycosylated selectin ligands, thereby blocking leukocyte rolling (Etzoni *et al.*, 1993).

In physiological inflammation, adhesion receptors and signalling molecules are transiently expressed, thus limiting leukocyte recruitment and avoiding tissue damage by leukocyte accumulation. In contrast, these molecules and signals may persist in pathological responses (Tab. 2). Pathologically upregulated leukocyte adhesion and activation results in tissue damage associated with a number of inflammatory diseases. Accumulated neutrophils cause tissue necrosis by uncontrolled release of reactive oxygen intermediates, proteolytic enzymes and by production of inflammatory cytokines, which further enhance the reaction.

1.4. Role of different domains for L-selectin function

The three selectin-family members have a homologous domain structure with a N-terminal C-type lectin domain, an EGF-like domain, a varying number of short consensus repeats (SCR), a transmembrane domain and a short cytoplasmic tail. The ligand binding specificity of L-selectin resides in the lectin domain, since a P-selectin chimera that carries the L-selectin lectin domain binds to the complex carbohydrate ligands polyphosphomannan ester (PPME) and fucoidan, which are not recognised by the wild type P-selectin but by L-selectin (Kansas *et al.*, 1991).

Cells expressing this chimera also bind to HEV in a frozen section assay, whereas cells expressing P-selectin do not. The importance of the lectin domain is also underlined by the high degree of conservation between species.

The EGF and SCR domains of L-selectin mainly seem to confer structural integrity, and may be substituted by the homologous domains of other selectins without altering binding specificity. The SCRs are also thought to contribute to a proper spacing of the selectin ligand binding domains from the cell surface (Kansas, 1992).

The short (17 amino acids) cytoplasmic domain of L-selectin displays a high degree of conversation between species, which points to a functional importance, but it differs from the other selectins. The juxtamembrane region is rich in basic amino acids. Three residues in the cytoplasmic tail may serve as potential phosphoryl acceptor sites: two serines (S377 and S380) in the central part of the domain, and the C-terminal tyrosine residue (Fig. 4).

The importance of the intracellular part for L-selectin function has been shown by deletion experiments, which implicated a connection to the cytoskeleton to be essential for the ability of L-selectin to support rolling (Kansas *et al.*, 1993 and see below). Other studies also demonstrate that L-selectin function is regulated by signalling events involving the cytoplasmic domain. Lineage specific signals that lead to activation of lymphocyte subsets induce a fast and transient increase in L-selectin ligand affinity (Spertini *et al.*, 1991). This observation points to the existence of an inside-out signalling mechanism similar to the events

leading to integrin activation. Several recent publications showed that L-selectin crosslinking with ligand or antibodies also leads to the generation of outside-in signals, which elicit a series of cellular responses in the leukocyte (Crockett-Torabi, 1998).
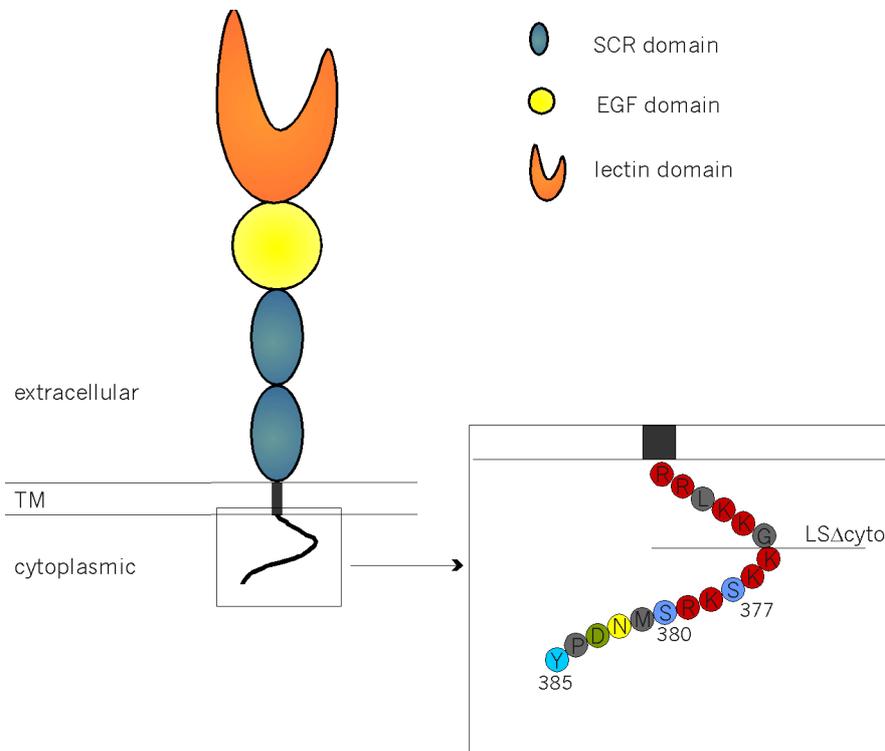
Fig. 4: Domain architecture of L-selectin.
Residues of the cytoplasmic domain are coloured according to their chemical properties: red: basic, green: acidic, grey: non-polar, yellow: polar, blue: putative phosphoryl acceptor sites. Position numbers of serine and tyrosine residues correspond to the full length protein, including the propeptide. LSΔcyto refers to the deletion mutant used in studies examining the role of the cytoplasmic domain for L-selectin function.

1.5. Intracellular interactions of L-selectin

1.5.1 Receptor positioning

Leukocytes possess a complex surface structure with ruffles and microvilli protruding away from the cell body. L-selectin is found selectively localised at the tips of microvilli which was shown to facilitate interactions with endothelial cells during rolling (Erlandsen *et al.*, 1993, von Andrian *et al.*, 1995). Other adhesion molecules involved in the initial binding to the vessel wall, like ESL-1, PSGL-1 and α_4-integrins, are also confined to microvilli (Berlin, *et al.*, 1995; Moore *et al.*, 1995; Steegmaier *et al.*, 1997), whereas β_2 integrins and CD44 are excluded from this structures and only found on the cell body (Erlandsen *et al.*, 1993).

The localisation of L-selectin is determined by the transmembrane and/or intracellular part of the molecules, since a CD44/L-selectin chimera with the ectodomain of CD44 replaced by that of L-selectin is positioned on the cell body, whereas the transmembrane and cytoplasmic domain of L-selectin direct the

CD44 extracellular part to microvilli (von Andrian *et al.*, 1995). The redistribution of the chimera to the cell body interferes with L-selectin function. Although cells transfected with chimeric molecule bound soluble ligand with an affinity comparable to cells expressing wild type L-selectin, they showed a significantly lower capture frequency under flow conditions on immobilised ligand, supporting the assumption that L-selectin presentation on the tip of microvilli is crucial for initial interaction.

The part of the transmembrane/cytoplasmic region that is responsible for proper receptor positioning has not yet been determined. Although partial deletion of the cytoplasmic domain affects the function of L-selectin, it does not change localisation on microvillous tips (Pavalko *et al.*, 1995 and see below). However, a role for the remaining intracellular residues of the deletion mutant in cytoskeletal association has been shown, so it appears possible that this region is involved in receptor positioning (Dwir, 2001). Alternatively, the transmembrane domain could participate in the process, since lateral interactions between membrane spanning molecules in the plasma membrane have been described for many receptors.

1.5.2 Connection of L-selectin to the cytoskeleton

For most transmembrane molecules involved in adhesion events, a constitutive or inducible connection to the cytoskeleton has been reported (Pavalko and Otey, 1994). This anchorage is thought to enable adhesion receptors to withstand the stress applied during interactions without being 'pulled out' of the cell membrane. First indications of a cytoskeletal association of L-selectin came from the finding that deletion of the C-terminal 11 amino acids severely impaired the ability of transfected cells to bind to HEV *in vitro* and roll on mesenterial vessel walls *in vivo*. Interestingly, the ligand recognition of the extracellular portion of the receptor was not affected by this deletion, because cells expressing the truncated L-selectin mutant (LΔcyto) still bound soluble ligand comparable to wild type cells (Kansas *et al.*, 1993).

L-selectin has been shown to bind the cytoplasmic actin-binding protein α-actinin, which links several transmembrane receptors to the actin cytoskeleton (Pavalko *et al.*, 1995). The association was found *in vitro* using purified proteins, demonstrating direct interaction of the two molecules. When L-selectin was precipitated from cell lysates, a complex of α-actinin and vinculin was detected, although vinculin alone did not bind to L-selectin *in vitro*. This indicates the formation of a cytoskeleton linker protein complex, with additional proteins associating indirectly with L-selectin via α-actinin. The deletion mutant LΔcyto did not associate with these cytoskeletal proteins, demonstrating that the C-terminal 11 amino acids are involved in the interaction.

Evans *et al.* (1999) showed that binding of GlyCAM to L-selectin or crosslinking with lectin-domain binding primary mAb and secondary Ab induced a redistribution of L-selectin to the detergent-insoluble cytoskeletal fraction. This points towards an inducible tight association of the cytoplasmic domain with the cytoskeleton. With the LΔcyto deletion mutant this redistribution to the cytoskeletal fraction could not be observed. Therefore, the connection is dependent on the membrane-distal part of the cytoplasmic tail, probably through

binding of the associated α-actinin to cytoskeletal elements. The interaction was not blocked with cytochalasin B, colchicine or nocodozole showing that *de novo* formation of actin filaments or microtubules is not required for interactions and that L-selectin rather associates with preformed cytoskeletal elements. Inhibitors of serine/threonine or tyrosine phosphorylation also do not block translocation to the detergent-insoluble fraction. The authors concluded that for initial interactions with ligand connection with the cytoskeleton is not required, since in resting cells, L-selectin, although constitutively associated with α-actinin, shows no tight anchorage to the cortical cytoskeleton. This connection is then induced rapidly after ligation of the receptor itself or alternatively by signals transduced in the inside of the cell. In this study, exposure of the cells to hyperthermia, which has been shown to have a profound effect on the actin based cytoskeleton and signal transduction events (Di *et al.*, 1997; Wang *et al.*, 1999), lead to L-selectin connection to the cytoskeleton and simultaneously to increased binding of lymphocytes to HEV sections (Wang *et al.*, 1998). These results indicate that L-selectin avidity can be regulated by physical interactions between L-selectin and the cortical actin-based cytoskeleton.

Further evidence for the existence of different levels of L-selectin adhesiveness came from investigation of L-selectin dependent tethering and rolling at high temporal resolution (Dwir *et al.*, 2001). This study demonstrated that initial tethering is almost normal in the LΔcyto mutant under moderate shear stress, but that conversion of transient tethers to stable rolling was significantly impaired. Increase in shear rate diminished the ability to support tethering and further reduced rolling mediated by the mutant receptor. The ability to support tethering was completely abolished by further truncation of the cytoplasmic domain by four amino acids, indicating that a anchoring mechanism independent of α-actinin is employed, which involves the membrane-proximal region of the cytoplasmic domain. Recently, members of the ezrin-radixin-moesin-(ERM)-family of cytoplasmic proteins have been reported to bind to the cytoplasmic domain of L-selectin in *in vitro* assays (Ivetic *et al.*, 2001). Affinity chromatography using peptides that span the complete intracellular part of L-selectin isolated moesin from phorbol ester-stimulated lymphocytes, whereas ezrin from both untreated and stimulated cells bound to the peptide. Mutational analysis demonstrated that an arginine residue in the membrane-proximal region is essential for interaction with both proteins. Although this interaction has not been confirmed in a cellular context, this results support the idea that, apart from α-actinin, other mechanisms for cytoskeleton anchorage might be involved in L-selectin dependent adhesion.

1.5.3 Cellular signal that regulate L-selectin function (inside-out-signalling)

Regulation of L-selectin affinity

Treatment of leukocyte populations with lineage-specific activating stimuli (Ab directed against the TCR-complex or CD2 for T-cells and G-CSF, G/M-CSF or TNFα for neutrophils) induced an increased L-selectin dependent binding of soluble ligand and attachment to HEV-sections (Spertini *et al.*, 1991). This

enhanced binding was not accompanied by an increase in surface expression of L-selectin, but was rather attributed to a raised affinity of the receptor for ligand. The upregulation of binding affinity is induced rapidly after stimulation and declines quickly due to shedding of L-selectin from the cell surface. The authors speculated that cellular activation induces a conformational change in the L-selectin molecule, leading to an increased affinity for ligand and therefore promoting attachment of leukocytes. The selective activation of leukocyte subpopulations with lineage-specific stimuli could contribute to the regulated trafficking of subsets to appropriate sites.

A possible molecular mechanism for this finding was provided by Haribabu *et al*. (1997), who showed that L-selectin was phosphorylated following chemoattractant-induced signalling events. In [^{32}P]orthophosphate labelled lymphoblastoid cell lines, L-selectin is constitutively phosphorylated at low levels and this phosphorylation is increased following treatment with phorbol ester. In cells co-transfected with L-selectin and receptors for fMLP, IL-8 or PAF, stimulation with the appropriate factor also induced L-selectin phosphorylation. This phosphorylation was abolished by mutation of the two serine residues present in the cytoplasmic tail of L-selectin, demonstrating the involvement of serine/threonine kinases. Pertussis toxin, which blocks the G-protein mediated signalling of chemoattractant receptors, also abolished L-selectin phosphorylation.

Chemoattractant receptor signals and phorbol ester are known to activate protein kinase C (PKC). When cells were treated with staurosporine, which strongly inhibits PKC activity (but also several other serine/threonine kinases), no L-selectin phosphorylation occurred.

Treatment with staurosporine also reduced both basal binding and increased binding following CD3-crosslinking to HEV frozen sections. These findings point towards a role of L-selectin phosphorylation on serine residues in regulation of L-selectin affinity.

Induction of L-selectin shedding

Shedding of L-selectin is another event regulated by intracellular transduced signals. Rapid proteolytic cleavage of L-selectin from the surface of both lymphocytes and granulocytes is observed following a multitude of activating stimuli, including the PKC activator PMA, cytokines, chemoattractants and crosslinking of various surface receptors (Kishimoto *et al*., 1989; Spertini *et al*., 1991a; Chao *et al*., 1997; Frey *et al*., 1997). Downregulation was also induced by crosslinking L-selectin itself with chemical agents or antibodies, indicating that L-selectin is shed during the physiological adhesion process (Palecanda *et al*., 1992). In accordance to this assumption, adhesion of neutrophils to activated endothelium induced a loss of L-selectin (Smith *et al*., 1991). By inhibitor and knock-out studies, the shedding protease has been characterised as an ADAM-metalloprotease identical with or closely related to TNFα-converting enzyme (TACE) (Bennett *et al*., 1996, Feehan *et al*., 1996, Preece *et al*., 1996, Peschon *et al*., 1998).

Several signalling pathways seem to contribute to L-selectin shedding, dependent on the stimulus used. In inhibitor studies, ligation of the Leu-13 surface antigen or L-selectin itself was found to induce shedding by a tyrosine kinase-dependent, but PKC-independent pathway, whereas cleavage following phorbol

ester and fMLP treatment or CD3 crosslinking is blocked by staurosporine but not influenced by tyrosine kinase inhibition (Alexander *et al.*, 2000; Frey *et al.*, 1997; Stoddart *et al.*, 1996). The MAP kinases ERK and p38 are also implicated in regulation of shedding of L-selectin as well as TGF-α and TNFα receptor, since activated mutants of these kinases induced shedding of the receptors, whereas inhibition of the kinases prevented proteolysis (Fan and Derynck, 1999).

The physiological significance of L-selectin shedding for leukocyte extravasation is still controversial. Inhibition of shedding using a metalloprotease inhibitor was found not to block neutrophil adhesion and transmigration using activated endothelium under flow conditions (Allport *et al.*, 1997). On the other hand, when L-selectin shedding is blocked, rolling has been observed to be slower, and the degree of β_2-integrin activation is strongly augmented, probably by longer exposure to activating signals at the vessel wall and/or enhanced signalling by L-selectin (Hafezi-Moghadam *et al.*, 2001).

L-selectin shedding has been shown to be regulated by binding of calmodulin to the cytoplasmic domain of L-selectin (Kahn *et al.,* 1998). This interaction was observed in *in vitro* assays with purified protein, demonstrating direct binding, and in co-precipitation experiments. When binding of calmodulin is blocked by an inhibitor, shedding of L-selectin is strongly enhanced. According to this study, calmodulin is bound to the intracellular tail in resting cells and is released when intracellular calcium levels rise following activation. This may cause a conformational change in the L-selectin molecule which allows the protease to access the cleavage site. Calmodulin binding involves the membrane-proximal part of the cytoplasmic domain, since point mutations in this region abrogated the interaction and increased L-selectin shedding in the absence of stimulus (Matala *et al.*, 2001).

1.5.4 Signal transduction initiated by L-selectin (outside-in-signalling)

Cellular activation

Studies of many adhesion receptors revealed that they do not merely have structural function, but serve as signal transducing molecules as well (reviewed by Aplin *et al.*, 1998). In accordance to this concept, L-selectin has been shown to initiate a number of signalling events upon ligand binding.

In neutrophils, treatment with activating factors like chemokines leads to a prolonged activation of NADPH oxidase, and, as a result, to the production of reactive oxygen intermediates like O_2^- and H_2O_2. This so-called oxidative burst is essential for the defence against invading pathogens. Crosslinking of neutrophil L-selectin also leads to cell activation characterised by production of reactive oxygen compounds and an increase in intracellular Ca^{2+} levels (Crockett-Torabi and Fantone, 1997). These events are preceded by tyrosine phosphorylation signals, since they are inhibited by genistein, a broad range inhibitor of tyrosine kinases. The L-selectin signal acts in synergy with activating cytokines and chemoattractants like TNFα and fMLP (Waddell *et al.*, 1994).

Interplay of L-selectin signalling with other activating factors is also seen in the receptors contribution to the functional upregulation of leukocyte integrins. In neutrophils, L-selectin crosslinking leads to upregulation of surface Mac-1 expression (Crockett-Torabi *et al.*, 1995). Mac-1-dependent binding of neutrophils to albumin-coated beads was induced by PAF and IL-8 as well as Ab against L-selectin (Simon *et al.*, 1995) and the effects of chemoattractants and receptor activation were additive (Tsang *et al.*, 1997). Integrin activation is accompanied by a change in cell shape due to actin polymerisation and by co-localisation of L-selectin with β_2 integrins (Simon *et al.*, 1999).

In lymphocytes, L-selectin ligation with GlyCAM-1 or Ab also induces functional activation of β_1 and β_2 integrins without increasing surface expression (Hwang *et al.*, 1996; Giblin *et al.*, 1997). T-cell adhesion to ICAM-1, VCAM and fibronectin following L-selectin crosslinking is strongly inhibited by tyrosine kinase inhibitors, but little affected by blocking of PKC signalling (Sikorski *et al.*, 1996).

Sulfatides, sulfated glycolipids which have been shown to bind to L-selectin, also elicit a calcium response in neutrophils, as well as increased levels of TNFα and IL-8 mRNA. Since cytokine expression is regulated on several levels and actual release of these factors from the cell has not been tested, the physiological implications of these findings remain unclear. The study further does not exclude involvement of other surface receptors in sulfatide-binding, as they have recently been identified (Ding *et al.*, 2000).

Initiation of signalling cascades by L-selectin

Activation of several cellular signal transduction pathways is induced by extracellular ligation of L-selectin. Antibody crosslinking of the receptor leads to increased tyrosine phosphorylation of a number of cellular proteins in neutrophils (Waddell *et al.*, 1995). One of these proteins was identified as MAP kinase, which also shows enhanced kinase activity after stimulation through L-selectin.

In lymphocytes, overall tyrosine phosphorylation is also enhanced by L-selectin ligation (Brenner *et al.*, 1996). Phosphorylated proteins have been shown to include L-selectin itself. This phosphorylation was dependent on the src-family kinase p56lck, since a T-cell line lacking this kinase does not show increased tyrosine phosphorylation and transfection of the cells with lck-cDNA reconstitutes phosphorylation. This lck-deficient cell line also showed impaired rolling behaviour on fucoidan which can be recovered by retransfection of lck (Brenner *et al.*, 1997b). Other signalling molecules showing enhanced activity following L-selectin ligation in an lck-dependent manner include the small G-proteins Ras and Rac, which might be involved in MAPK-activation. The stress-activated kinase JNK (jun N-terminal kinase), but not the related p38 kinase is also activated by L-selectin signalling. The activation of JNK occurs via lck and the small G-proteins Rac1/2, since inhibition of this signal pathways prevented activation of JNK (Brenner *et al.*, 1997c). Cytoskeletal rearrangement following L-selectin ligation was likewise inhibited by suppression of Rac2 as well as Ras (Brenner *et al.*, 1997a). Rac2 is known to be located in signalling pathways downstream of Ras and has been shown to be able to regulate JNK.
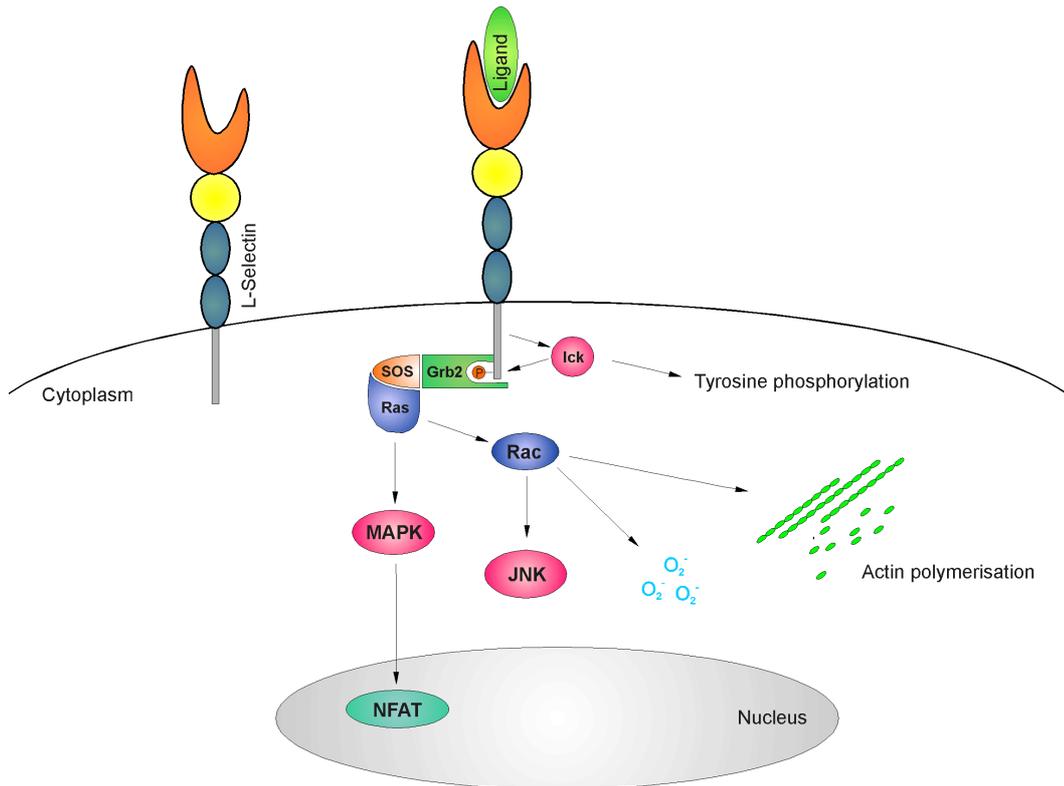
Fig. 5: L-selectin mediated signal transduction events
Crosslinking of L-selectin with antibodies or ligand binding to the receptor have been shown to elicit a wide range of cellular responses.

Furthermore, L-selectin induces activity of the neutral sphingomyelinase, leading to ceramide release, which acts as a second messenger for various cellular responses (Brenner *et al.*, 1998).

Although several signalling intermediates involved in transducing signals initiated by L-selectin ligand binding have been identified, the details of the generation of this signals, especially events occurring close to the receptor, are yet unclear.

1.6. Aim of this study

Ligand-binding to L-selectin has been shown to induce a series of cellular events, demonstrating that this adhesion receptor acts as a signal transducing molecule. Whereas several "downstream" signalling events, including kinase activation, generation of second messengers and cytoskeletal rearrangements, have been described, nothing is known about the generation and transduction of the signal on the level of L-selectin itself.

This study therefore aims to characterise proteins that interact with the cytoplasmic domain of L-selectin and might serve as signal transducers, thereby linking the receptor to intracellular signalling cascades. For identification of intracellular interaction partners, different approaches are used:

- genetic screening:
  The yeast two-hybrid method provides the means to screen cDNA-libraries for interaction partners in the context of a living cell. This method offers the advantage of direct access to the cDNA encoding the interacting protein, facilitating further characterisation of the interaction.
- biochemical isolation using a recombinant fusion protein that contains the intracellular part of L-selectin:
  affinity purification of proteins binding to the cytoplasmic part and identification by mass spectroscopy.

The cytoplasmic domain of L-selectin does not possess any intrinsic enzymatic activity or known protein-protein interaction motifs, but contains three potential phosphorylation acceptor sites: the C-terminal tyrosine 385 and two serines at positions 377 and 380. L-selectin has been reported to be phosphorylated at both tyrosine and serine residues, although the kinases involved are unknown. An objective of this study is the elucidation of phosphorylation mechanisms leading to phosphorylation of residues in the cytoplasmic part of L-selectin. This includes *in vitro* phosphorylation studies with purified kinases and characterisation of kinases interacting with the cytoplasmic tail.

Since phosphorylation of proteins involved in signal transduction processes frequently create binding sites for other signalling molecules, this work further aims at the identification of phosphorylation-dependent binding partners. For this, affinity isolation with *in vitro* phosphorylated fusion proteins will be performed.