L-SELECTIN, AN ADHESION MOLECULE: EXPERIMENTAL MEASUREMENT OF ITS SHEDDING AND POSSIBLE CLINICAL IMPLICATIONS

MASTER IN BIOMEDICINE AND MOLECULAR BIOLOGY

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Performed by:

Jaime Azpiazu Saiz

72099110-K

Director:

Marcos López Hoyos

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Dr. Marcos López Hoyos, Associate Professor of Inmunology at the University of Cantabria and Head of the Inmunology Department at University Hospital Marques de Valdecilla

AUTHORIZES Mr. Jaime Azpiazu Saiz to present his work to obtain the Master,degree in Biomedicine and Molecular Biology, and entitled

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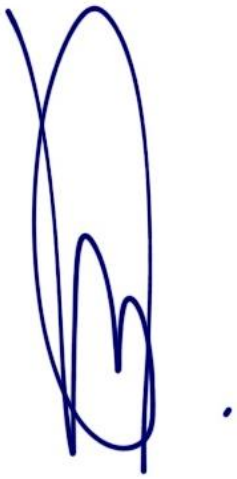
Fdo.: Marcos López Hoyos



Abstract

L-selectin (CD62L) is a type I transmembrane glycoprotein and cell adhesion molecule that is expressed in most circulating leukocytes. L-selectin is broadly characterized as an anchor/bearing receptor. There is currently emerging evidence suggesting that L-selectin has a role in regulating monocyte protrusion during transendothelial migration (TEM). The lectin domain is N-terminal calcium-dependent (type C) interacts with numerous glycans, important to name sialyl Lewis X (sLex) for anchoring/coiling and proteoglycans for endothelial transmigration. The short cytoplasmic tail of 17 amino acids is responsible for adhesion. The ability of leukocytes to migrate from the periphery to tissues is a critical step in the immune response, with several adhesion molecules being involved in this process. This molecule is expressed on the surface of lymphocytes, neutrophils, monocytes, eosinophils, hematopoietic precursor cells, and immature thymocytes. L-selectin is a highly glycosylated protein of 95-105 kD in neutrophils and 74 kD in lymphocytes. It is involved in lymphocyte migration to peripheral lymph nodes through interaction with GlyCAM-110 and in the adhesion of lymphocytes, neutrophils and monocytes to the endothelium activated by cytokines at sites of inflammation. Several L-selectin ligands have been identified on endothelial cells, GlyCAM-1, CD34 and MAdCAM-1, all containing glycosylated mucin domains. The soluble form of L-selectin (sL-selectin) is present in plasma due to metalloproteinase-mediated cleavage of L-selectin expressed on the cell surface. The soluble form retains bioactivity and at high concentrations can inhibit the binding of lymphocytes to the endothelium, suggesting their possible role *in vivo*. While sL-selectin can be detected in the circulation of healthy individuals from 0.7 to 1.5ug/ml, increased levels have been reported in patients with sepsis, inflammatory, autoimmune diseases and in leukemias. An indirect approach to measure sL-selectin is by means of the shedding of CD62L from the membrane of leukocytes. In this work we set up a method to measure it *in vitro* in order to apply it in the clinical management in patients with suspicion of immunodeficiency

Keywords: *Diapedesis, Inmunodefficiency,* L-selectin*, Lymphocytes*, Shedding*.*

GENERAL INDEX

1. I NTRODUCTION 7

1.The steps in the process of inflammation and extravascular migration…….8

1.1 What are the cellular and molecular elements involved in Acute Inflammatory Response that cause all the events of inflammation?.....8

1.1.1. Vascular events……8

1.1.2. Cellular events……12

2. Importance of adhesion molecules and main types……14

2.1. How are these molecules regulated to induce leukocyte adhesion in inflammation?.....15

3. Selectins into L-selectin……..16

3.1. L-selectin gene expression ……..16

3.2 Organization of L-Selectin……18

3.3 Regulation of L-Selectin Protein Expression……23

4. Role of sL-selectin and involvement in inflammatory pathology……...25

5. Hypothesis……26

6.Patients and Methods……..26

7. METHODOLOGY……27

8. CONCLUSSIONS……29

9. BIBLIOGRAPHY……..30

Figure index

Figure 1: 17

Figure 2: 22

1. INTRODUCTION

1.1 The steps in the process of inflammation and extravascular migration.

There are various stimuli that can cause tissue injury, either exogenous or endogenous, that can cause the inflammatory response (IR) in the vascularized connective tissue. This vascular reaction results in the infiltration and accumulation of fluid and leukocytes in the extravascular tissues.(1,2)

The five cardinal signs of inflammation are: flushing, tumour, heat, pain, and functional impotence (Virchow's sign). (3)

The IR is closely related to the repair process. IR is useful for destroying, attenuating, or keeping the damaging agent localized, and simultaneously initiates a series of events that can determine the healing or reconstruction of injured tissue. Hence, inflammation is considered to be primarily a protective response, and in the absence of this process, infections would spread uncontrollably, wounds would never heal, and injured organs would present suppurative lesions permanently. However, in certain situations, such as allergic reactions and chronic diseases, the inflammatory process constitutes the basic pathogenic mechanism. (1-4)

The inflammation presents two well differentiated phases: acute and chronic. Acute inflammation has a relatively brief evolution; Its fundamental characteristics are the exudation of liquid and plasma proteins (edema), and the migration of leukocytes (mainly neutrophils). Chronic inflammation lasts longer and is characterized by proliferation of blood vessels, fibrosis, and tissue necrosis. (5-7)

1.1 What are the cellular and molecular elements involved in Acute Inflammatory Response that cause all the events of inflammation?

There are several elements involved in Acute Inflammatory Response (AIR). Among them, we will consider: plasma, circulating cells (neutrophils, monocytes, eosinophils, basophils and lymphocytes), blood vessels and connective tissue cells (mast cells, fibroblasts and macrophages) and extracellular matrix components (collagen, elastin, adhesion glycoproteins such as fibronectin, laminin, non-fibrillar collagen, tenascin, and proteoglycans) of the connective tissue. In addition, the cellular and vascular responses of AIR are mediated by chemical factors from plasma or cells that are activated by the inflammatory stimulus itself. These mediators act independently, sequentially or in combination, and in later phases they amplify the IR and influence its evolution. Necrotic cells and tissues can also trigger the formation of chemical mediators. (5-7)

1.1.1. Vascular events

Secondary to injury, whatever its nature, there is an inconsistent and transient period of arteriolar vasoconstriction. Then, certain amounts of histamine appear at the damaged site, causing vasodilation and active hyperemia (increased blood flow in the area of the lesion), which causes redness and an increase in temperature.(3)

Histamine is the mediator of the acute phase and is stored in granules of mast cells, basophils and platelets. It is secreted in response to physical injuries, such as trauma, cold or heat; or in the presence of inflammatory agents such as complement molecules (C3a, C5a), lysosomal proteins, or interleukins (IL) IL1, IL8. (4-6)

Another mediator that may appear at the injured site is nitric oxide (NO), released in this case by endothelial cells in response to the injurious stimulus. Its main action is vasodilation through relaxation of the smooth muscle of the vascular wall. In addition to endothelial cells, it is also produced by specific neurons and macrophages. It is synthesized from L-arginine, molecular

oxygen, nicotinamide-adenine dinucleotide reduced phosphate (NADPH) and other cofactors, by action of the enzyme nitric oxide synthase (NOS). Endothelial NOS is rapidly activated by increased cytosolic calcium. (6-7)

Later, a slowdown in local blood flow occurs, secondary to a progressive increase in vascular permeability with extravasation of fluid and an increase in blood viscosity in vessels of smaller size, determining the establishment of the state of vascular stasis. As stasis evolves, peripheral orientation (marginalization) of leukocytes occurs, attaching to the endothelium, crossing the vascular wall, and targeting the interstitium.

The vascular events that occur in inflammation can be summarized in the following order:

1. Arteriolar and capillary vasodilation, which causes capillaries and venules to open, induced by the action of different mediators on vascular smooth muscle, mainly histamine and nitric oxide.

2. Increased blood flow through the arterioles, which is the cause of the appearance of erythema (flushing) at the site of inflammation.

3. Increased permeability of the microvasculature, leakage of an inflammatory exudate into extravascular tissues, and the appearance of inflammatory edema.

4. Abnormal and excessive accumulation of blood, the leakage of fluid causes an increase in the viscosity of the blood, which increases the concentration of red blood cells (venous congestion).

5. Decreased blood speed in small vessels (blood stasis).

6. Peripheral accumulation of leukocytes, marginalization and leukocyte paving.

7. At the same time, endothelial cells are activated by the mediators of inflammation, expressing molecules in their membranes that favor the adhesion of leukocytes, mainly polymorphonuclear neutrophils (PMN).

8. Leukocyte passage (PMN first, followed by macrophages) from the vessels to the interstitium: cell migration, with formation of the inflammatory infiltrate

The alteration of the permeability constitutes the main characteristic and of greater specificity of the AIR, causing the profuse exudate towards the interstitium. Loss of plasma proteins reduces intravascular osmotic pressure but increase in interstitium. After vasodilation, intravascular hydrostatic pressure increased and leads to a significant leakage and accumulation of fluid in the interstitial tissue, eventually forming edema.

The increase in vascular permeability is generated by several mechanisms, which can occur simultaneously.

* First, the formation of openings between endothelial cells of venules. This mechanism is activated by histamine, bradykinin, leukotrienes, substance P, and other types of chemical mediators. This form of filtration only affects venules. The process appears to be mediated by intracellular agonist mechanisms, where endothelial cell contractile proteins phosphorylation is involved, which is known as an immediate response. Bradykinin (another mediator) is released by activation of the kinin system. This system generates vasoactive peptides from plasma proteins called kininogens and through specific proteases called kallikreins; one of these vasoactive peptides is bradykinin, a powerful inducer of increased vascular permeability, as well as pain. The kinin system is activated by Hageman's factor (XII of coagulation), which in turn is activated by injury to the vascular wall and expression of negative charges (collagen and basement membrane). (6) Leukotrienes (lipid mediator) are products of the metabolism of arachidonic acid (AA). AA is a 20-carbon polyunsaturated fatty acid (5, 8, 11, 14-eicosatetraenoic acid), found inside cells esterified with membrane phospholipids. Upon mechanical, chemical (platelet activating factor, C5a) and physical stimuli, cellular phospholipases are activated, which liberate AA from its union with the membrane. AA metabolites (eicosanoids) are synthesized by two classes of

enzymes: cyclooxygenases (obtaining prostaglandin and thromboxanes) and lipoxygenases (obtaining leukotrienes and lipoxins). (7) Prostaglandin G2 (PGG2) is the main metabolite of the cyclooxygenase pathway. In mast cells it is found together with prostaglandins E2 (PGE2) and F2α (PGF2α), which have a wider distribution. PGG2 leads to vasodilation and enhance the formation of edema. In the case of substance P it must be stated that it plays an important role in the initiation of IR. It constitutes a neuropeptide (abundant in nerve fibers of the lung and the gastrointestinal system). It is a powerful mediator of the increase in vascular permeability; It is also involved in the transmission of painful signals, regulation of blood pressure and stimulation of secretion by immune and endocrine cells. (8-10)

* Cytoskeleton reorganization (endothelial retraction): Interleukin-1 (IL-1), tumor necrosis factor (TNF) and interferon gamma (IFN-y) are involved in this process, as well as hypoxia. It is known as a late response. IL-1 and TNF share many biological properties, and are produced by activated macrophages. Its secretion can be stimulated by physical injuries, endotoxins, immunocomplexes and toxins. (11-12) Regarding the increase of permeability, these cytokines induce the synthesis of endothelial adhesion molecules and other cytokines, chemokines, growth factors, eicosanoids and NO. They also induce the synthesis of enzymes associated with the remodelling of the matrix, are prothrombotic factors, and stimulate the synthesis and activity of collagenase enzymes. TNF also induces aggregation and priming of neutrophils, releasing proteolytic enzymes, contributing to tissue damage. (13)

* Increased trancytosis: The transport of fluids and proteins through the endothelial cells themselves (and not between them) can be carried out through channels that are formed from interconnected vacuoles and uncoated vesicles (called vesiculovacuolar organelle). Vascular endothelial growth factor (VEGF) appears to stimulate the number and size of these channels.

* Direct endothelial injury, with necrosis and detachment of endothelial cells: necrosis of endothelial cells causes their separation from the vessel wall, thereby creating an opening in it. It can occur in severe wounds, such as burns, or by the toxic action of microbes that directly affect the endothelium. PMNs

that adhere to endothelial cells can also damage them. In this case, fluid loss continues until a thrombus forms or damage is repaired. It is known as a prolonged immediate response. (14)

* Leukocyte-mediated endothelial lesion: leukocytes attached to the endothelium release toxic forms of oxygen and proteolytic enzymes that end up damaging the endothelium, with a consequent increase in permeability.

1.1.2. Cellular events:

One of the characteristic and important function of inflammation is the contribution of leukocytes to the area of injury. Leukocytes phagocytize pathogens, destroy bacteria and microorganisms, and degrade necrotic tissue, but they can also prolong tissue damage by releasing enzymes, chemical mediators, and reactive oxygen species (oxygen free radicals, RLO). (6 -10) The sequence of events that occur from the leukocytes leaving the vascular lumen until they reach the interstitial tissue (extravasation) can be divided into:

1. Intravascular: marginalization, rolling and adhesion.

   When blood flow is normal, erythrocytes and leukocytes in vessels remain confined to a central axial column. As the speed of blood flow decreases in the early stages of inflammation, due to increased vascular permeability; hemodynamic conditions are modified (shear forces on the wall decrease) and a greater number of leukocytes is located towards the periphery, along the endothelial surface. This leukocyte accumulation process.

2 Transmigration through the endothelium (diapedesis).

The leukocytes direct their pseudopodia toward the junctions between endothelial cells, enter tightly through them, and are located between the endothelial cell and the basement membrane. Finally they cross the basement membrane and exit into the extravascular space. This exit mechanism is used

by neutrophils, monocytes, lymphocytes, eosinophils, and basophils. It is known that leukocyte adhesion and transmigration are primarily determined by the binding of complementary adhesion molecules to the surface of leukocytes and endothelial cells, and that chemical mediators (chemotactic factors and certain cytokines) influence these processes by regulating expression. surface and binding intensity of these adhesion molecules

3 Migration in the interstitial tissues towards a chemotactic stimulus.

According to a chemical gradient. All granulocytes, monocytes and to a lesser extent lymphocytes, respond to chemotactic stimuli with different degrees of speed.

Exogenous and endogenous substances can act as chemotactic factors: Exogenous: bacterial products. Endogenous: components of the complement system, especially C5a, products of the lipoxygenase pathway, mainly leukotriene B and cytokines

The binding of chemotactic agents to specific receptors located on the leukocyte cell membrane leads to reactions that produce an increase in cytosolic calcium, a factor that triggers the assembly of the contractile elements responsible for cell movement.

2. Importance of adhesion molecules and main types.

It is well-known that adhesion and transmigration are fundamentally determined by the binding of complementary adhesion molecules to the surface of leukocytes and endothelial cells, and that chemotactic factors and some cytokines influence these processes by regulating surface expression and intensity of fixation of these adhesion molecules. (9-12)

Inflammation requires leukocytes to pass from the bloodstream into the tissues, basically the neutrophils and monocytes are the ones that move to the inflamed tissues in response to local stimuli. In the first phase of cell adhesion, selectins intervene reversibly, and leukocytes are rolled by the inflamed endothelium. In a second phase, the binding or arrest of leukocytes to the endothelium and the activation of neutrophils by the complement fragment C5a, PAF (platelet-activating factor), and interleukin 8 (IL-8), as well as by the FMLP peptide (n-formyl-methionyl-leucyl-phenylalanine). The next step is a firm binding of the cell to the endothelium, intervening for this purpose adhesion molecules that belong to the immunoglobulin superfamily, such as ICAM-1 (intercellular adhesion molecules-1) and VCAM (vascular adhesion molecules-1). And lastly, a change in the shape of the cell occurs, allowing extravasation.

Some authors also call this cascade as the metastatic cascade, because the same steps of the adhesion cascade are used for the dissemination of certain tumor cells, which are in the bloodstream and pass through the endothelium. (11-14)

The adhesion receptors correspond to:

Selectins: they have an extracellular N-terminal region related to sugar-binding lectins, E-selectin (confined to the endothelium), P-selectin (present in the endothelium and platelets), and L-selectin (in leukocytes). They bind to the sialylated forms of the oligosaccharides, which in turn are covalently linked to mucin-type glycoproteins.

Immunoglobulins superfamily: ICAM-1 (intercellular adhesion molecule type 1) and VCAM-1 (vascular adhesion molecule type 1); both are endothelially adherent and interact with the leukocyte integrins.

Integrins: glycoproteins of transmembrane adhesion. The main integrin-like receptors are: for ICAM-1 the beta integrins (LFA-1 and MAC-1; for VCAM-1 the integrins α4β1 (VLA-4) and α4β7. (9-14)

2.1. How are these molecules regulated to induce leukocyte adhesion in inflammation?

* Redistribution of adhesion molecules to the cell surface: P-selectin is normally found in the membrane of intracytoplasmic granules (Weibel-Palade bodies). Being stimulated by histamine, thrombin, and platelet activating factor (PAF), P-selectin is redistributed to the cell surface, where it can fix leukocytes, it is important in the initial phase of leukocyte bearing. (7 -14) PAF is a chemical mediator derived from phospholipids. From the chemical point of view it is an acetyl-glyceryl-ether-phosphorylcholine. It is produced by mast cells, basophils, neutrophils, monocytes, macrophages, endotheliocytes, and platelets. It performs its effects through a receptor coupled to a G protein (activation of the second messenger). In addition to inducing leukocyte adhesion to the endothelium, it also causes degranulation, chemotaxis, oxidative burst, platelet aggregation, and in high concentrations causes vasoconstriction and bronchoconstriction. (13-14)

* Induction of adhesion molecules on the endothelium: cytokines (IL-1 and TNF) induce the synthesis and expression on surfaces of endothelial adhesion molecules. E-selectin, which does not normally exist in the endothelium, is induced by IL-1 and TNF, and acts as a mediator of the adhesion of neutrophils, monocytes, and certain lymphocytes by binding to their receptors.12 These cytokines also activate ICAM-1 and VCAM-1. (12-14)

* Increased binding intensity: This is the most important mechanism for the attachment of integrins. For LFA-1 to bind to ICAM-1, neutrophils must be activated, so that LFA-1 goes from a low-affinity state to a high-affinity state for ICAM-1, because the integrin undergoes a transformational type transformation. The main agents that cause this leukocyte activation are chemokines, made by the endothelium or by other cells that come from the area of injury. During inflammation, the increased affinity of LFA-1, together with the increased expression of ICAM-1, determines the necessary conditions for a tight leukocyte-endothelium binding to occur. The VCAM-1 interaction seems to be a necessary element for the subsequent transmigration through the endothelium. (10-14)

3.1. L-selectin gene expression

Located on the long arm of chromosome 1 (1q24.2), it is organized in tandem with the members of its family (in the order: L-, P- and E-selectin). Its gene consists of ten exons spanning a 21.0 kb region. This human gene is regulated by FOX01 (15,16), and chromosomal immunoprecipitation has been able to identify some others (Mzf1, Klf2, Sp1, Ets1 and Irf1) (17). The molecular weight of L-selectin differs between cell types, ranging from 70 kDa in lymphocytes to 100 kDa in neutrophils, and depends on the cell-type specific glycosylation. The altered glycosylation patterns in L-selectin may promote cell-specific functions, although this is not yet perfectly described. L-selectin is made up of structural compartments: a calcium-dependent lectin domain (type C) (CTLD), an EGF-like domain, two repeated complement-like sequences, and an extracellular cleavage site. (18)

Different L-selectin splices have been classified into murine models and human models. The mouse cell gene was made up of 9 exons. The two splice variants, classified as L-selectin-v1 and Lselectin-v2, have an additional exon, created between exons 7 and 8. The different splices

share the first sequence of 49 bp, while L- selectin-v2 spans an additional 51 bp. that's immediately 3 'to this region. These splice variants are characterized by having longer cytoplasmic tails (WT = 17 aa; v1 = 30 aa; v2 = 32aa; see Figure 1). Overall, levels of L-selectin-v1 and -v2 mRNA make up 23% of total L-selectin mRNA, so its impact on trafficking and endogenous leukocyte signalling is not fully understood. The human splice variant does not have exon 7, it is in charge of encoding the transmembrane domain. Thus, transcripts that lack this exon are secreted and soluble. Patients with rheumatic diseases have a higher prevalence of splice variant transcription (16).

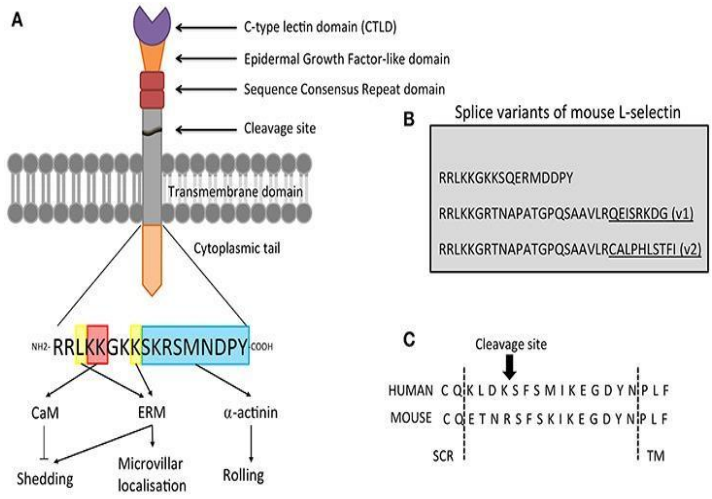


Figure 1**.** *Organization of the L-selectin domain. (L-. When going from N-terminal to C-terminal, it is broken down into: C-type lectin domain (CTLD), epidermal growth factor (EGF), two sequence consensus repeat domains (SCR), section of cleavage, transmembrane domain and a 17 amino acid cytoplasmic tail. The amino acid sequence of the cytoplasmic tail of human L-selectin is shown, highlighting the amino acids that support binding to calmodulin (CaM), ERM proteins and alpha-actinin (A) All three sequences belong to the mouse L-selectin tail (note that the mouse L-selectin tail has a single serine at position 364, whereas the human L-selectin tail has a residue of additional serine at position 367. Conservation of the sequence in the proximal region of the membrane that supports binding to ERM and CaM (RRLKKG) is 100% conserved. The amino acid sequences of two L-*

selectin splice variants of mouse (v1 and v2) are provided in the sequences below. The underlined residues represent sequences that are unique to v1 and v2 (B). Amino acid sequences surrounding the mouse and human L-selectin cleavage site. The arrow indicates the cleavage position. TM, transmembrane domain; SCR, consensus sequence repeated region (C)(17)

3.2 Organization of L-Selectin

L-selectin is similar in extracellular domains to E- and P-selectin (Figure 1A). On the other hand, the cytoplasmic tails of members of the selectin family are not similar, probably transmitting unique intracellular signals.

We begin with the possession of **CTLD at the N-terminus** (19), which belongs to a large superfamily of metazoan proteins with different functions (17-18). It has the function of anchoring and rolling the leukocytes by working together with the minimal tetrasaccharide determinant, sLex (14). A unique ability of selectins is to stabilize bond life with conformational changes in the CTLD determined by an external force such as hydrodynamic shear capacity. E-selectin mutation product showed the existence of coordinated links between Ca2 + and amino acid residues within the upper face of the CTLD stabilizing interaction (15,16). Fucose 3 and 4-hydroxyl groups in sLex also form a coordinated Ca2 + bond, which collectively stabilize the selectin / ligand binding (17). L-selectin binding to sLex is characterized by a critical shear stress threshold condition (typically between 0.3 and 1.0 dynes per cm2). L-selectin binding depends on a 'catch' and 'slip' binding mechanism, where optimal shear stress conditions can expose more of the ligand binding domain and increase adhesiveness, and where an increase in pulling force eventually exceeds the threshold of the catch link (18).

X-ray crystallographic evidence of selectins reveals that the CTLD folds into an EGF-like region of the domain, linked by a hydrogen bond between Y37 and N138 (20). This interaction between domains restricts L-selectin to a less adhesive conformation while in circulation. The functional significance of this interaction was characterized in neutrophils, where the decoupling of the Y37 / N138 hydrogen bond increases the useful life of the bond, which is manifested

in a greater priming of neutrophils within the circulation (20-23). Neutrophils carrying an N138G knock-in mutation within L-selectin showed increased bacterial destruction and worsened results in sterile lesion models. Both phenotypes were directly related to the increased preparation of neutrophils, confirming the causality of the knock-in mutation. This mode of "mechanochemistry" has been suggested to exist in other L-selectin expressing immune cells (24).

SCR Domains are found variably and determined SCR (in humans L-selectin has 2, E-selectin has 6 and P-selectin has 9), which have homology to complement regulatory proteins. They are called "sushi domains" and are present in several different cell adhesion molecules (24). The function of the SCR serves to distance the CTLD from the plasma membrane, seeking, the extension beyond other cell adhesion molecules, to support the anchorage and rolling behavior. The possibility studied of why L-selectin has less than the members of its family is due to morphology because it is anchored to microvilli, which gives it an advantageous position for anchorage under flow.

The Cleavage domain (Figure 1C) is essential since L-selectin undergoes ectodomain shedding at a specific membrane proximal location, eleven amino acids above the transmembrane domain, this fact occurs between K321 and S322. (25) Alanine scan mutations surrounding the cleavage site would suggest redundancy. Deletion of multiple amino acids suggests that the actual distance of the cleavage site from the plasma membrane is more important (26). ADAM17-inactivated mice reveal the accumulation of L-selectin on the surface of neutrophils and monocytes, suggesting that both induced ectodomain turnover and elimination are mediated by a similar enzyme. Due to the relaxed specificity of the L-selectin cleavage site, it is difficult to definitively state that both basal and activated K321 / S322 detachment. We are in the position that the binding of calmodulin to the cytoplasmic domain of L-selectin regulates the conformation of the cleavage site (27)).

The cytoplasmic Tail in composed of intracellular proteins, including: alpha-actinin (28), calmodulin ezrin, moesin (29), protein kinase C (PKC) isoenzymes (30) and μ1alpha / AP-1 (31). Given the size of the L-selectin

tail, not all of these proteins can bind simultaneously, but they are likely to interact under strong spatio-temporal constraints, for example during anchoring, rolling, firm adhesion, and TEM. More of this will be discussed in the section below.

Removing the 16 C-terminal amino acids from the tail of L-selectin can dramatically affect its lateral mobility along the plane of the plasma membrane, caused by the lack of anchorage to the underlying cortical actin-based cytoskeleton (32). Such large truncation can interrupt immobilization dynamics under flow conditions (33) and reduce the efficiency of L-selectin release.

Alpha-Actinin is In charge of crosslinking classic actin filaments. There are isoforms (1 to 4), each encoded by a different gene and differentially expressed. Isoforms 1 and 4 are expressed in non-muscle cells and have molecular weights of approximately 100 kDa when highlighted in polyacrylamide. In contrast, isoforms 2 and 3 are shown in skeletal and cardiac muscle cells. Deletion of the 11 C-terminal amino acids from L-selectin (called ‘LΔCyto ') nullifies the interaction with alpha actinin binding (35). Injection of cell lines expressing LΔCyto Lselectin into the circulation of rats revealed a significant reduction in rolling efficiency within the inflamed mesenteric parenchyma venules (36). They claim the requirement of the L-selectin cytoplasmic tail in the regulation of rolling interactions. However, little is known about how alpha-actinin interacts with L-selectin and whether the interaction is regulated by phosphorylation (serine / tyrosine) of the L-selectin tail or the production of secondary messengers (eg, Ca2 +). Alpha-actinin isoforms 1 and 4 possess CaM-type EF hands, which can bind Ca2 + and inhibit actin crosslinking activity. Interestingly, isoforms 1 and 4 expressed in smooth muscle cells (as in chicken gizzard) exist as splice variants of the EF domain that are insensitive to Ca2 + (37). Pavalko et al. They probably use Ca2 + sensitive and insensitive forms of alpha-actinin. It is currently unclear whether Ca2 + binding to alpha-actinin could play an active role in

regulating L-selectin binding. Despite its conserved identity with CaM, the EF-hand of alpha-actinin is not believed to interact with L-selectin since the amino acids that support the binding of CaM and alpha-actinin are found at opposite ends of the tail of L -selectin (37). Non-muscular alpha-actinin is known to bind to other tails of cell adhesion molecules and has been extensively reviewed elsewhere (38).

Calmodulin (CaM) is the protein responsible for adhering to 18 kDa calcium, identified by immunoprecipitation, and was subsequently confirmed by *in vitro* solid phase binding assays. It is at a resting state when Cam joins L-Selectin. Upon cell stimulation, CaM dissociates to promote an allosteric change at the cleavage site to drive ectodomain clearance by ADAM17. Serine 364, but not S367, in human L-selectin has been shown to be responsible for dissociation of CaM from L-selectin. In fact, the mutation of S364 to alanine significantly reduces forbol myristate acetate-induced release (PMA). (39)

(ERM) Proteins are classified as 3-membered cytoskeletal proteins weighing 75-80 kDa. They all contain very similar domains: a globular N-terminal domain, which belongs to the ezrin-radixinmoesin (FERM) of band 4.1, a central alpha-helical domain and an acid actin binding domain (see Figure 2A). The main role of ERM is to serve as linkers of the membrane cytoskeleton. Moreover, it is increasingly evident that they play essential roles in mediating signal transduction. Overexpression of L-selectin in fibroblasts can lead to the formation of filopodia-like extensions. ERM proteins have the ability to actively participate in the formation of microvilli (40,41). The ERM conformation when inactive is folding, phosphatidylinositol 4,5bisphosphate allows it to open (Figure 2B) (42-44).

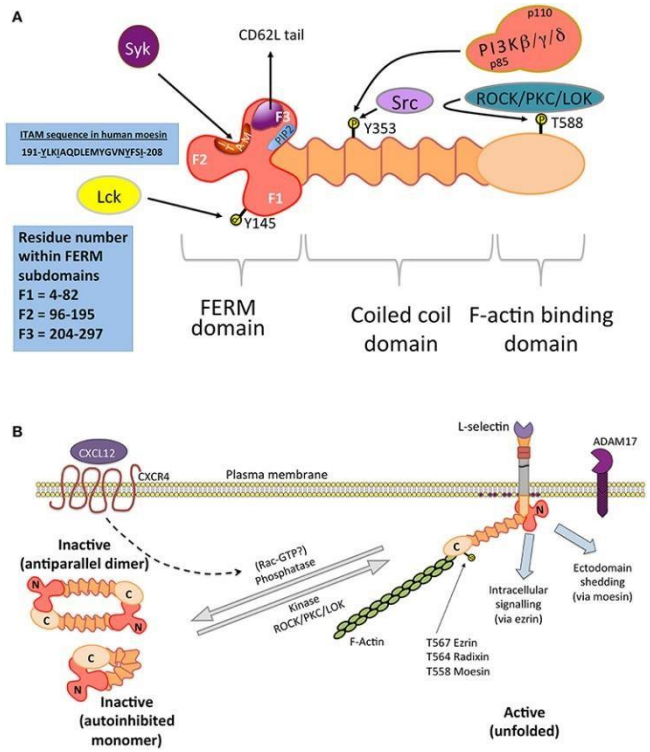


Figure 2 (A) *Organization of the ERM domain: X-ray crystallographic studies determine the N-terminal domain. FERM contains a globular clover leaf shape (determined by 3 different subdomains) (the blue box indicates the residue numbers that make up each subdomain. (B) The FERM domain contains several interaction sites: a cryptic ITAM (the sequence is shown, which can recruit Syk, a PIP2 binding site and a region responsible for L-selectin binding. Tyrosine kinase Lck is responsible for Y145 phosphorylation of the FERM domain in T cells. Phosphorylation of Src from Y353 in the ezrin core coil domain can lead to the recruitment of PI3K class I, via the p85 regulatory subunit. are specifically classified as membrane / cytoskeleton crosslinkers. Recent evidence specifically that ezrin may be involved in the regulation of intracellular signaling while moesin regulates L-selectin clustering before ADAM17 removes its ectodomain. A pathway where ERMs are inactivated in T cells has been detected, where binding of CXCR4 to CXCL12 leads to rapid dephosphorylation and collapse of ERM microvillar*

AP-1 Adaptin (μ1A) is composed of clathrin-coated vesicles, was identified in classical deployable experiments and then verified by protein-protein interaction (47).

μ1A drives the transport of *de novo* synthesized L-selectin from the trans Golgi network to the plasma membrane. The mode of interaction induced by PMA could suggest that MRF is internalized by virtue of the μ1A binding, once L-selectin removal is complete. Full-length L-selectin can be internalized by μ1A and taken to an endosomal recycling compartment for re-expression back to the plasma membrane at a later stage during lymph node trafficking (45).

3.3 Regulation of L-Selectin Protein Expression

Modification of the actin cytoskeleton and elevated CD62L of the bone marrow of a neonate clarifies that L-selectin is one of the first surface markers that is expressed in lymphoid-primed hematopoietic stem cells (46). A similar observation in mice clarifies that L-selectin plays an essential role in trafficking and differentiation of stem cells. CD10 and CD62Lhi cells can differentiate into dendritic cells, monocytes, NK cells, B cells, and T cells. L-selectin expressed in these early progenitor cells is likely to be necessary for trafficking from the bone marrow to the peripheral lymphoid organs. It is not perfectly clear whether subsequent differentiation depends on L-selectin. (47,48)

The leukocyte expresses on average 50,000–70,000 L-selectin molecules in the plasma membrane. Numerous reports have shown that L-selectin is targeted at finger-like projections called microvilli, which increases the effectiveness of anchoring during recruitment (49). Protein expression of L-selectin is constitutive in most circulating leukocytes, and it slowly turns around in the plasma membrane through a process of detachment of the ectodomain (commonly called "shedding"). A variety of artificial or physiological agonists of cell activation can, within minutes, promote robust L-selectin release in numerous leukocyte subtypes). Zinc-dependent metalloproteinase, a disintegrin and metalloproteinase (ADAM) 17, is the main enzyme responsible for the release of L-selectin in leukocytes. However, ADAM 8 and 10 have also

been reported to cleave L-selectin in specific settings (59,60). From a clinical perspective, the soluble circulating form of L-selectin (released as a consequence of ectodomain clearance) is sometimes used as a plasma / serum surrogate biomarker for leukocyte activity triggered during acute or chronic inflammation (50-53). Similarly, leukocytes expressing low levels of surface L-selectin (examined by flow cytometric analysis) are a classic indicator of cellular activation (54). With the paradox that a drop in soluble L-selectin can also be detected in certain diseases, such as sepsis (55). The drop in detectable soluble L-selectin may be due to its adsorption to luminal vascular ligands that are regulated during sepsis. Alternatively, L-selectin could be cleaved from transmigrated neutrophils within the abluminal/non-luminal regions of the vessels. Although soluble L-selectin may compete for cell-associated L-selectin, little is understood about how the two forms dampen leukocyte recruitment during inflammation. Soluble L-selectin is detected in the plasma of healthy humans (0.7-1.5 μg per ml), suggesting that cell-associated L-selectin is cleaved from circulating leukocytes at rest at low levels. Indeed, mouse neutrophils lacking ADAM17 express above-average surface levels of L-selectin (56). This phenomenon is also observed when synthetic broad-spectrum inhibitors of ADAM17 are used, or when the L-selectin cleavage site is mutated and becomes "sheddase resistant" (57-60). The way that L-selectin expression is regulated, either at the translational or post-translational level, will be unique in different leukocyte subsets. For example, the lifespan of a central memory T cell (TCM) far exceeds the lifespan of a neutrophil (i.e. 6 weeks compared to 8 days, respectively) . Aging neutrophils have diminishing levels of L-selectin, which contrasts with MTC .The sections below provide some examples of how Lselectin is expressed in different leukocyte subsets and the impact this might have on the behavior of immune cells (60)

4. Role of L-selectin and involvement in inflammatory pathology.

L-selectin is expressed in circulating neutrophils, monocytes, and eosinophils, and in most virgin B lymphocytes and T lymphocytes. L-selectin has been implicated in the adhesion process of neutrophils with activated endothelial cells, but not with quiescent endothelial cells, indicating that the ligand for L-selectin is not constitutively present in endothelial cells. L-selectin-dependent leukocyte-endothelium interactions do not require leukocyte activation, as this molecule is constitutively expressed. In fact, the activation of leukocytes by inflammatory mediators or cytokines results in a decrease in the expression of L-selectin in the plasma membrane by shedding of this molecule.

A characteristic that distinguishes L-selectin from the other members of his family is the way special regulation; various stimuli thatinclude chemoattractants, cytokines, and esters of phorbol induce the release of a fragment soluble of said molecule. Stimulating lymphocytes from BALB / c mice with phorbol esters, Preece et al. identified a metalloproteinase responsible zinc-dependent membrane of L-selectin proteolysis. He physiological significance of this phenomenon is not still know exactly, but it has been proposed that loss of L-selectin would allow leukocytes detach from the luminal surface of the vascular wall and initiate migration between endothelial cells; Furthermore, the soluble fragments of this molecule would bind to its ligands in endothelial cells adjacent to the site of inflammation and this shape would block the binding of other granulocytes to restrict their access to the area of injury.GlyCAM-1, found in the upper endothelial venules of the lymph nodes.

L-Selectine Ligands

CD34, found in endothelial cells.

MadCAM-1, found in endothelial cells of lymphoid tissue associated with the intestine.

PSGL-1 binds with low affinity.(70-75).

5. Hypothesis and objective

L-selectin shedding was observed with granulocytes from all of the interleukin-1 receptor-associated kinase-4-deficient patients on activation with agonists of Toll-like receptors 1/2, 2/6, 4, 7, and 8 and with granulocytes from all of the UNC-93B-deficient patients on activation with agonists of Toll-like receptors 7 and 8. All of the healthy controls responded to these stimuli.

L-Selectin shedding may be an indirect and easy method to study defects in immunodeficient patients in which defects in the phagocyte system are suspected. The laboratory work proposed was to set up the laboratory method to measure L-selectin shedding in peripheral blood mononuclear cells and implement such a practice to the Immunology Laboratory routine.

6. Patients and Methods

Heparin-treated blood was taken from 8 healthy control subjects after informed consent. We incubated 100 uL of blood for 1 hour without stimulation or with TLR4 agonist (LPS), or phorbolmyristyl acetate (PMA) at 2 mg/uL at 37°C in a humidified atmosphere containing 5% CO_2.. For all of the incubations the cells were first incubated with 10 g/mL of polymyxin B (Sigma, P-4932) for 20 minutes. (74-76)

The procedure is obtained starting with the heparin tubes to which the LPS and PMA stimuli are added, which are incubated 1 h at 37ºC

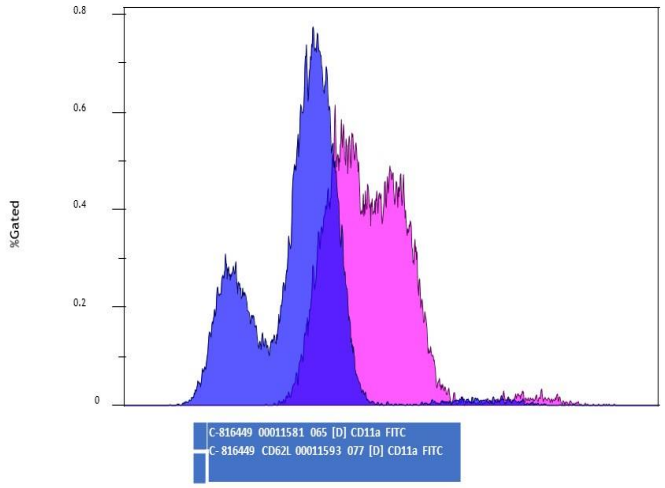
Once incubated, we added 500 ul of lysis buffer and then incubated 10 min at room temperature.

Subsequently we introduced 500 ul of Isoflow buffer (Beckman Coulter) and incubated at room temperature for 5 min.

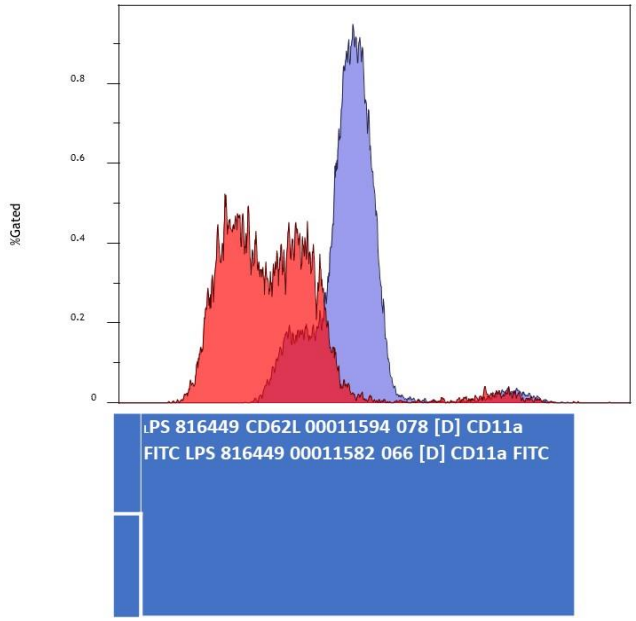
Subsequently, we splited in two tubes and in one of them carried the CD62L. We then incubated 30 min and then obtained the cell pellet after centrifugation. The pellet was resuspended in 500 ul PBS to be acquired in the Flow Cytometer (Aquios,, Beckman Coulter).

7. Results

- Expression without stimulus



- Expression with stimulation of TLR4



- Expression with PMA + Ionomicin

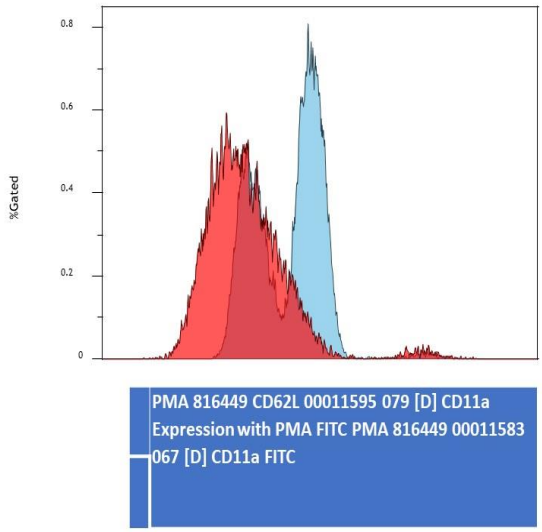


Figure is a representative histogram of one of the 8 healthy controls studied. Above histogram represents the signal of CD62L staining in unstimulated granulocytes (magenta) in comparison with the signal of unstimulated cells without antibody staining (autofluorescence in blue).

The middle figure shows the CD62L staining in unstimulated granulocytes (magenta) and the CD62L shedding after stimulation with LPS (in red).

The lower histogram represents the CD62L staining in unstimulated cells (blue) compared with the CD62L shedding in stimulated granulocytes with PMA + Ionomycin (red).

Unfortunately, I must say that the Covid-19 pandemia has prevented me from far exceeding the test that is intended to be a future objective of study. The experiments with the 8 subjects were performed in the laboratory just before the outbreak of the pandemia. This would be the basis to follow-up setting-up the laboratory protocol to introduce it the clinical routine testing of patients with phagocyte defects suspicion in the Primary Immunodeficiency Clinics.

8.CONCLUSIONS

Understanding the role of l-selectin is key to understanding its role in the acute inflammation reaction and subsequently applying the relevant technique that can adhere to the possibility of immunodeficiencies.

The situation of the coronavirus will not prevent the project from continuing and it can be developed in the near future.

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