L-Selectin – a signalling receptor for lipopolysaccharide

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The activation of leukocytes by bacterial cell-wall lipopolysaccharide contributes to the pathogenesis of septic shock. We propose that in neutrophils, and possibly other leukocytes, L-selectin can act as a low-affinity lipopolysaccharide receptor. Inhibitors of L-selectin may therefore be of therapeutic value in treating this life-threatening condition.

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Lipopolysaccharide (LPS), an outer membrane component of Gram negative bacteria, is a potent activator of leukocytes and endothelial cells. LPS stimulates cells to express a series of genes coding for immunoregulatory, inflammatory and cytotoxic molecules, such as tissue factor, tumor necrosis factor α (TNF-α), interleukin (IL)-1 and IL-6. The excessive secretion of these mediators results in pathophysiological effects in humans, causing symptoms of sepsis or septic shock. Characteristic symptoms of sepsis are fever, hypothermia and an increased white blood cell count; septic shock is a form of acute circulatory failure. Septic shock is a life-threatening condition, considered to be the commonest cause of death within intensive care units. An estimated 500,000 people are affected annually by this syndrome in the USA alone. Mortality is typically 40–60% within 28 days of diagnosis, despite antibiotic therapy and state-of-the-art intensive care medicine. Septic shock patients die of cardiovascular prolapse and multiple organ failure exacerbated by a locally inadequate blood supply (ischaemia) and an infiltration of leukocytes in the tissue [1]. It is known that *in vivo* a concentration of < 1ng/ml of LPS is sufficient to induce the symptoms of sepsis, although higher concentrations of LPS (>100 ng/ml) are required to produce symptoms of septic shock [1]. How does LPS produce such dramatic effects?

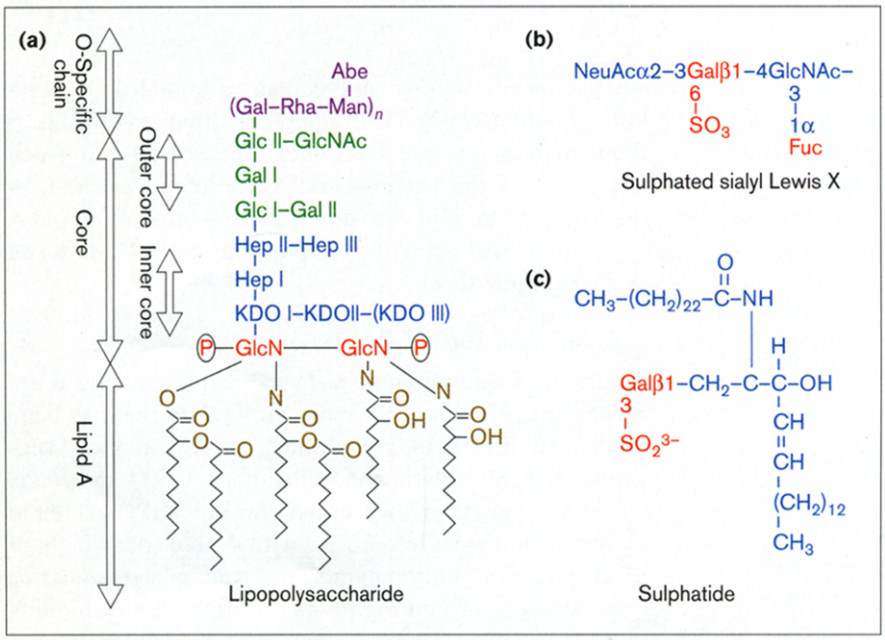
LPS characteristically consists of a polysaccharide region with a covalently bound lipid A component (Figure 1a). The polysaccharide region consists of an O-specific chain, an outer core of sugar moieties and a conserved inner-core oligosaccharide [2]. The O-specific chain is characteristic for a given LPS and varies between different species and serotypes of bacteria. The inner core of a variety of LPSs are chemically similar and contain 2-keto-3-deoxyoctulosonic acid (KDO). The lipophilic lipid A domain is thought to anchor the LPS onto the bacterial outer cell wall. Lysis of the bacterial wall is therefore required for the exposure of lipid A to other binding proteins. Lipid A is the principal structure responsible for LPS-induced sepsis or septic shock.

Lipopolysaccharide-binding proteins

Several LPS-binding proteins have been characterised (for review see [3]). The only molecule to date that has been implicated directly in LPS binding and LPS-induced activation of host cells is the cell-surface CD14 molecule. CD14 is a glycerophosphatidyl inositol (GPI)-anchored plasma membrane glycoprotein with a molecular weight of 53 kDa. It is present on monocytes, macrophages and, to a lesser extent, on neutrophils [4,5]. High-affinity binding of LPS to CD14 requires the presentation of LPS as a complex with serum LPS-binding protein (LBP). The subsequent signal transduction mechanisms leading to cell activation are not well understood. Because CD14 is a GPI-anchored cell-surface molecule, it is not itself thought to mediate signal transduction [5]. Patients suffering from paroxysomal nocturnal hemoglobinuremia are unable to form GPI anchors and therefore lack cell-surface CD14 and yet monocytes from these patients can be activated to secrete cytokines by LPS (with or without LBP) [6,7]. In addition, it has been shown that the LPS antagonists, lipid IVA and *Rhodobacter sphaeroides* lipid A (RSLA), inhibit LPS-induced cellular signalling by interacting with cell-surface protein(s) distinct from CD14 [8]. Furthermore, macrophages isolated from mice lacking the CD14 gene (CD14 knockout), have been shown to respond to LPS, but only at higher concentrations of LPS [9,10]. In the CD14 gene-knockout animals it was shown that the macrophage interaction with low concentrations of LPS (< 10 ng/ml) was CD14 dependent. At higher concentrations of LPS, no difference in TNF-α secretion was observed between macrophages from CD14 knockout and wild-type mice. These observations point to the existence of CD14-independent signalling mechanism(s) for LPS-induced cell activation [10]. Results obtained from the CD14-knockout animals are consistent with anti-CD14 antibodies studies in mice. It is known that, *in vivo*, high concentrations of LPS (> 100 ng/ml) can stimulate monocytes and neutrophils to produce cytokines without the involvement of CD14/LBP. Depletion of serum LBP by anti-LBP antibodies had little effect on the survival rate of mice injected with high doses of LPS [11,12].

CD14 also exists in a soluble form in plasma at a concentration of 2–6 μg/ml [13]. Soluble CD14 can bind LPS

Figure 1



The structure of carbohydrate and glycolipid ligands for L-selectin. **(a)** Lipopolysaccharide; **(b)** sulphated sialyl Lewis X; and **(c)** sulphatide. The likely binding site for L-selectin on each ligand is shown in red.

without a requirement for LBP [13,14]. The soluble CD14/LPS complex has been shown to bind to CD14-negative cells, for example endothelial cells, and to induce the secretion of cytokines from these cells [13,14]. Thus, CD14 is clearly not the only component required for LPS signalling, and the identity of the second component of the signalling receptor has, until recently, remained a mystery.

Selectins

Selectins are cell-surface integral membrane glycoproteins reported to be involved in leukocyte trafficking, thrombosis and inflammation. The three known selectins (E-selectin, P-selectin and L-selectin) are characterised by an amino-terminal carbohydrate recognition domain (CRD) followed by an epidermal growth-factor domain, two to nine complement control protein domains, a single membrane-spanning region and a cytoplasmic carboxy-terminal cytoplasmic domain (Figure 2). E-selectin is expressed principally on endothelium in response to inflammatory stimuli such as IL-1, TNF-α and bacterial lipopolysaccharides. P-selectin expression is also induced on endothelium and platelets in response to inflammatory responses. E-selectin expression is induced at the level of transcription, but P-selectin is stored in the α-granules and Weibel–Palade bodies of platelets and endothelium. L-selectin is expressed on essentially all blood leukocytes (for review, see [15]).

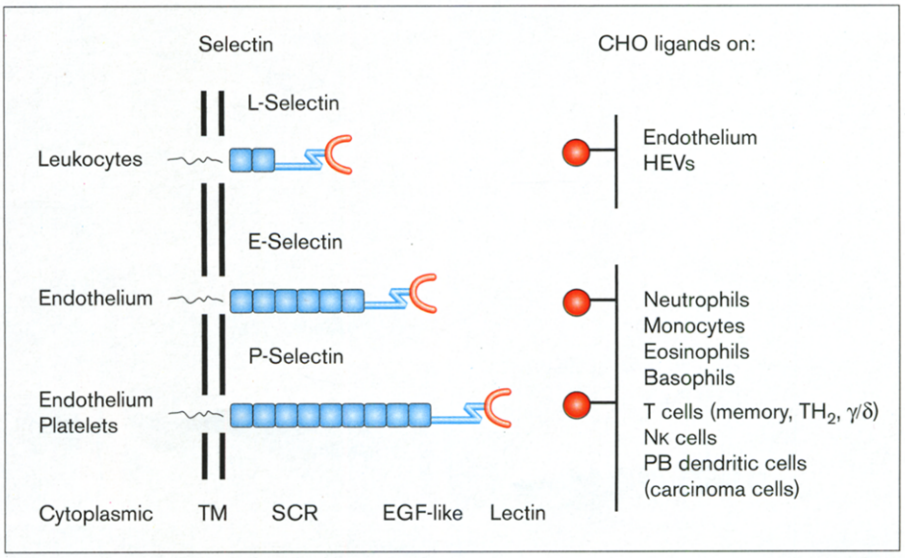
The generation of mice lacking individual selectins and *in vivo* studies with anti-selectin antibodies have provided insight into the physiological role of selectin-mediated adhesion (for review, see [16]). These data indicate that L-selectin and P-selectin mediate the initial capturing of leukocytes from the streaming blood, while the synergistic action of L-selectin and E-selectin or P-selectin is required for optimal and stable leukocyte rolling (the first stage in the process by which leukocytes exit blood vessels near a site of infection; selectins provide weak adhesion to the endothelial cell wall, allowing the leukocyte to roll along it).

On the basis of either direct binding or inhibition experiments, it has been shown that selectins share a common feature in recognising selectin ligands. The three selectins bind to fucosylated, sialylated oligosaccharides, such as sialyl Lewis X (sLe^x, NeuAcα2–3,Galβ1–4(α1–3Fuc) GlcNAc) in the presence of Ca^{2+} ions (Figure 1b). Although the binding affinity of selectins for sLe^x is in the low millimolar range, it has been suggested that higher avidity is achieved either through multiple binding or through the interaction of selectin (P-selectin or L-selectin) with a sulphated side group on the carbohydrate or protein backbone. P-selectin and L-selectin have also been reported to bind to heparan sulphate, glycosaminoglycans and sulphated glycolipids. These ligands lack sialic acid and fucose and can interact with selectins in the presence of EDTA, suggesting that they bind at a site distinct from the classical Ca^{2+}-dependent carbohydrate recognition domain [15].

Malhotra *et al.* [17] recently reported that charged phospholipids, such as cardiolipin and phosphatidylserine, bound to L-selectin in the presence of EDTA. This binding was shown to take place with a specific site on the C-type lectin domain of L-selectin incorporating the basic sequence KKNKE. A similar sequence (KRGK) has also been suggested to be a binding site for heparan sulphate on β-chemokines, for example MIP-1α [18]. It is likely that

Figure 2

A schematic representation of the domain structure of the three selectins, and the cells that express them. The carbohydrate (CHO) ligands for L-selectin are expressed on endothelium; most leukocytes express ligands for E-selectin and P-selectin. Leukocytes bind to endothelium using L-selectin to bind to the carbohydrates expressed on endothelium; in contrast, neutrophils and monocytes use carbohydrate structures to bind to the E-selectin or P-selectin expressed on the endothelium.



the Ca^{2+}-independent binding of L-selectin to charged lipids or glycolipids takes place through the phospholipid-binding site. Thus, it seemed possible that L-selectin might also bind to other lipid-linked, negatively charged structures, such as LPS.

L-Selectin as a signalling receptor

Evidence has been accumulating that L-selectin is also a signalling receptor. Sulphatides are 3-sulphated galactocerebrosides (a sulphate group is esterified on position 3 of the galactose; Figure 1c); they interact with several proteins, including laminin, thrombospondin, von Willebrand factor, properdin, antistasin and gp120 [19] .Recently, it has been reported that these compounds bind to L-selectin or P-selectin but not to E-selectin [20,21]. Ligation of cell-surface L-selectin on neutrophils with sulphatides or cross-linking with anti-L-selectin antibodies has been reported to induce transmembrane signalling [22]. Interaction of sulphatides or anti-L-selectin antibodies with neutrophils triggers an increase in intracellular free calcium, the release of oxygen radicals and an enhanced expression of cytokine mRNA (such as TNF-α and IL-8) [22,23]. This effect is dependent on the sulphation of the galactose ring, as non-sulphated galactocerebrosides did not bind to L-selectin and were also non-stimulatory. Binding of sulphatides to L-selectin or P-selectin is Ca^{2+} independent and it has been suggested that the sulphatides interact with L-selectin through a site distinct from the carbohydrate-binding site. We suggest that sulphatides interact with the anionic binding site on L-selectin in a manner similar to charged phospholipids [17].

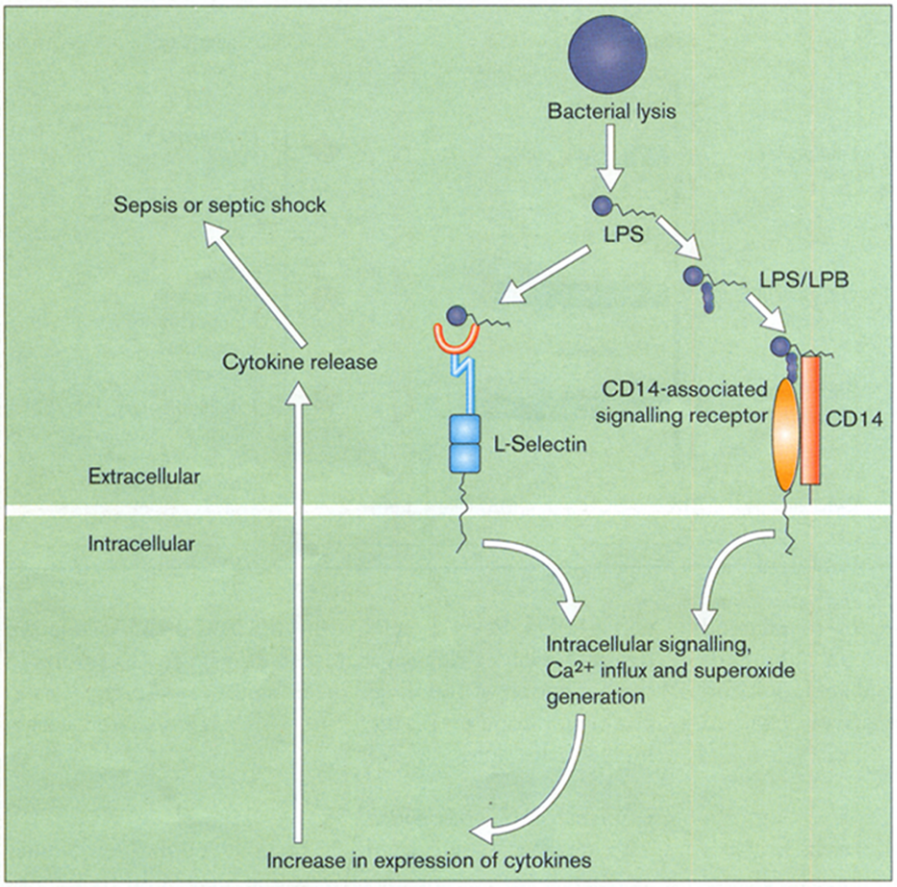
Further evidence that L-selectin can mediate signalling comes from studies of lymphocyte trafficking [24,25]. Hwang and coworkers [25] reported that the treatment of naive ($CD45RA^+$) T cells with GlyCAM-1 (an L-selectin ligand, which carries sulphated sLe^x; Figure 1b) results in the activation of the T cell, as detected by the expression of a neoepitope on β2-integrins which is associated with a high-avidity state.

L-Selectin and septic shock

The idea that L-selectin might be involved in the mortality associated with septic shock was first suggested by experiments performed by Tedder *et al.* [26]. High doses of LPS (40 μg of LPS per g of body weight) were injected into L-selectin-deficient and wild-type mice. In the wild-type mice 90% mortality was observed within 24 h, whereas in the L-selectin-deficient mice 90% of mice were still alive after 24 h. The majority (60%) of L-selectin-deficient mice survived the LPS-induced septic shock and appeared normal after four days. Tedder *et al.* suggested that resistance to LPS-induced mortality in L-selectin-deficient mice could be due to impaired recruitment and accumulation of leukocytes in tissues. But it has recently become clear that there may be another explanation for these findings. We recently reported that L-selectin binds to LPS isolated from several bacteria [27]. Both the binding of LPS to neutrophils and the LPS-induced activation of neutrophils was inhibited by anti-L-selectin antibodies. Our results indicate that L-selectin has a dual role: first as an adhesion molecule involved in leukocyte rolling, and second as a signalling receptor for LPS.

Further evidence for the role of L-selectin in LPS-induced signalling was provided by Higashi *et al.* [19] who reported that LPS-induced TNF-α secretion from the human monocyte cell line, THP-1, was inhibited by sulphatide in a dose-dependent manner. As discussed above, sulphatides bind to L-selectin and at higher concentrations (400 μg/ml)

Figure 3



A proposed model for the role of L-selectin and CD14 in lipopolysaccharide (LPS)-induced cytokine release from leukocytes. Bacterial lysis releases LPS, which binds either to LPS-binding protein (LBP) or directly to L-selectin. The LPS/LBP complex can bind to the glycerophosphatidyl inositol (GPI)-anchored receptor CD14, and this complex in turn can bind to an unidentified signalling receptor, which may or may not be L-selectin. Both L-selectin and the unidentified signalling receptor trigger intracellular events that lead to Ca^{2+} influx and eventually to cytokine release, hence to sepsis or septic shock.

can induce L-selectin-mediated transmembrane signalling. Due to the hydrophobic lipid sidechain, sulphatides at higher concentration tend to form micelles in water. It has been suggested that micelles of sulphatides can cross-link cell-surface L-selectin and mediate cell activation. But at lower concentrations (<100 μg/ml), sulphatides exist primarily in a monomeric form in which they can act as antagonists of L-selectin [19]. Higashi *et al.* [19] also reported that pretreatment of mice with sulphatide inhibited LPS-induced acute mortality, hypotension and the increase in levels of serum TNF-α. The degree of protection against LPS-induced mortality offered by sulphatide pretreatment *in vivo* was very similar to that reported by Tedder *et al.* [26]. Furthermore, rabbits pre-treated with anti-L-selectin antibodies were also resistant to LPS-induced septic shock [19]. The data from Higashi *et al.* [19] support our interpretation that L-selectin is a receptor for LPS and that inhibition of the interaction between L-selectin and LPS could be beneficial in treating severe symptoms associated with septic shock.

Further indirect evidence for the involvement of L-selectin in septic shock was provided by Donnelly *et al.* [28]. It is well established that the cross-linking of cell-surface L-selectin and the subsequent activation of leukocytes causes proteolytic cleavage and shedding of L-selectin [29]. Donnelly *et al.* [28] found a significant correlation between plasma levels of soluble L-selectin and the propensity to develop adult respiratory distress syndrome (ARDS). Patients with ARDS were suffering from multiple trauma, pancreatitis and perforated bowel. Their findings indicated that patients who subsequently developed ARDS had low levels of soluble L-selectin compared with patients who did not develop ARDS. It is likely that the patients with high levels of soluble L-selectin are resistant to ARDS due to inhibitory effects of soluble L-selectin on the interaction of LPS with leukocytes [28]. In future it will be useful to establish whether low levels of soluble L-selectin could be used as an indicator for the risk of progressing from sepsis to septic shock in patients.

We therefore propose that L-selectin has a dual role: first as an adhesion molecule involved in leukocyte rolling, and second as a signalling receptor for LPS. CD14 in the presence of LBP is a high-affinity receptor for LPS. At low concentrations of LPS, the binding of LPS to leukocytes is mainly through LBP/CD14 and thus the presence of LBP and CD14 is necessary for the activation of leukocytes. As

CD14 is not considered to be a signalling molecule, it has been suggested that CD14-associates with a distinct signalling receptor. It is possible that L-selectin could be the CD14-associated low-affinity signalling receptor. But at higher concentrations, LPS binds directly with L-selectin and does not require cooperativity between CD14 and L-selectin. In Figure 3, we propose a model for the role of L-selectin in LPS-induced cellular responses. These findings suggest that inhibitors of L-selectin may be of therapeutic value in treating the life-threatening condition septic shock.

In our opinion, future research should be aimed at developing specific inhibitors for LPS–L-selectin binding. The inhibitor(s) should act as an antagonist and not an agonist of L-selectin-mediated signalling. Higashi *et al.* [19] have shown that sulphatides, depending on concentration and their tendency to form micelles, can act as an agonist or antagonist of L-selectin-mediated signalling. One possible approach is to synthesise sulphatides with less tendency to form micelles, for example by modifying the lipid acyl chains. Another approach would be to search for inhibitors of L-selectin through screening or rationale design based on the putative anionic binding site [17] for LPS on L-selectin. An antagonist of L-selectin-mediated signalling will be useful both towards proving our hypothesis and towards a lead for a therapeutically useful drug.

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