

Novel aspects in the regulation of the leukocyte adhesion cascade

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Summary

Leukocyte recruitment plays a major role in the immune response to infectious pathogens and during inflammatory and autoimmune disorders. The process of leukocyte extravasation from the blood into the inflamed tissue requires a complex cascade of adhesive events between the leukocytes and the endothelium including leukocyte rolling, adhesion and trans-endothelial migration. Leukocyte-endothelial interactions are

mediated by tightly regulated binding interactions between adhesion receptors on both cells. In this regard, leukocyte adhesion onto the endothelium is governed by leukocyte integrins and their endothelial counter-receptors of the immunoglobulin superfamily. The present review will focus on novel aspects with respect to the modulation of the leukocyte adhesion cascade.

Keywords

Adhesion receptors, cell-cell interactions, endothelial cells, inflammation, integrins

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The multistep process of leukocyte recruitment

Leukocyte recruitment is crucial in the course of infection, in inflammatory disorders, such as atherosclerosis, as well as in autoimmune diseases, such as in psoriasis, rheumatoid arthritis, vasculitis and chronic lung diseases (1). Leukocyte extravasation comprises several adhesive steps including (i) the initial selectin-dependent rolling and tethering of the leukocytes, (ii) the chemokine-induced leukocyte activation, (iii) the integrin-mediated firm adhesion and (iv) the transendothelial migration of leukocytes, which can take place in both a paracellular and a transcellular manner. Finally leukocytes migrate in the extracellular matrix (1–11).

Rolling adhesions function as a “brake” to slow down the flowing leukocytes. The transient and reversible rolling interactions between leukocytes and the endothelium are mediated by weak binding between the E-, P- or L-selectin with their carbohydrate ligands, such as P-selectin glycoprotein-1 (PSGL-1) (12). PSGL-1 on leukocytes binds to both endothelial E- and P-selectin. P-selectin is stored in Weibel-Palade bodies, and inflammatory stimuli induce its rapid exposure on the apical endothelial surface, whereas E-selectin is newly synthesised upon pro-inflammatory stimulation (12). Besides mediating rolling and tethering, the P-selectin / PSGL-1 and the E-selectin /

PSGL-1 interactions play also an important role in the slow rolling process (13, 14). Recent evidence has pointed to the involvement of integrins, and particularly of the β 2-integrin lymphocyte function antigen-1 (LFA-1) in slow rolling adhesions that promote firm arrest from rolling (15, 16). Thus, the slow rolling process represents the transition between rolling and firm adhesion, where these two adhesive events functionally merge.

During the step of chemokine-induced activation, the low-affinity, selectin-dependent interaction is transformed into the high-affinity, integrin-mediated firm adhesion of leukocytes to the endothelium by activating signals derived from chemokines (17). The firm arrest of leukocytes to endothelial cells is mediated by interactions between leukocyte integrins, such as VLA-4 (α 4 β 1), α 4 β 7-integrin, Mac-1 (α M β 2) and LFA-1 (α L β 2), and their endothelial counter-receptors of the immunoglobulin superfamily, such as the intercellular adhesion molecules (ICAM), the vascular cell adhesion molecule-1 (VCAM-1), or the receptor for advanced glycation endproducts (RAGE), which are usually upregulated on the inflamed endothelium (3, 18, 19). Whereas VCAM-1 interacts with VLA-4, β 2-integrins (including LFA-1 and Mac-1) bind to ICAM1–5 (3, 18, 20). ICAM-1 and ICAM-2 are the mostly studied β 2-integrin ligands on the endothelium; ICAM-3, ICAM-4, and ICAM-5 are expressed in leukocytes, red cells and brain neurons, respectively (20–24).

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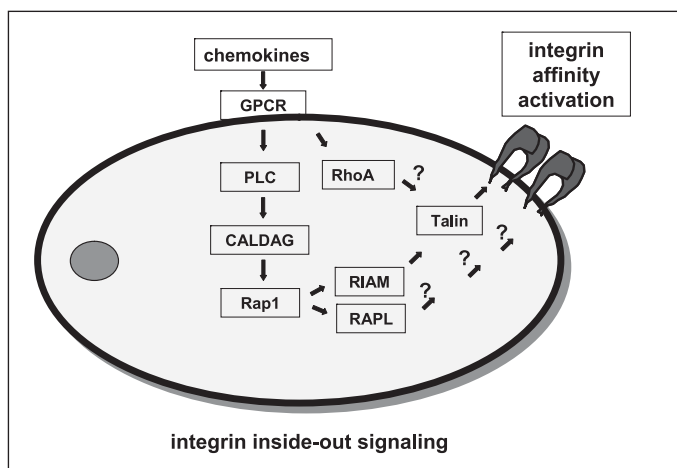


Figure 1: Inside-out signalling activation of leukocyte integrins. The contact of rolling leukocytes with the endothelium allows leukocytes to sense chemokines, presented on the apical endothelial cell membrane, which triggers integrin activation leading to the binding of the ligands on the endothelial cell surface and providing firm adhesion. Chemokines bind to their G-Protein coupled receptors (GPCR) thereby triggering the activation of PLC and CALDAG GEF1. CALDAG GEF1 activates in turn the small GTPase Rap1, which associates with effector proteins such as RIAM and RAPL inducing integrin activation. Specifically, RIAM associates with the cytoskeletal protein talin, which binds to the cytoplasmic tail of β -integrin subunits leading to separation of the cytoplasmic tails. This results in conformational alterations of the extracellular domains of the integrins, which adopt the intermediate or high-affinity conformation. Besides Rap1, also RhoA-signaling is involved in the chemokine-induced increase of integrin affinity in leukocytes.

After their firm adhesion, leukocytes move slowly over the endothelial cell surface using their integrins Mac-1 and LFA-1, a process called crawling or locomotion (8, 25) until they reach an area appropriate for transmigration. Transendothelial migration (also designated as diapedesis) may take place at the intercellular junctions, i.e. in a paracellular manner, or through the endothelial cell body, i.e. in a transcellular manner (26–28). The preference of the one pathway over the other is not clear and may depend on the level of the proinflammatory stimulation of the endothelial cells (29). The process of transmigration has drawn a lot of attention recently. Here, we are going to briefly summarize the players of transmigration, since this process was recently reviewed elsewhere (11).

Besides their importance in leukocyte-endothelial adhesion, ICAM-1 and ICAM-2 participate in leukocyte transendothelial migration as well. During diapedesis, ICAM-1 has been identified in microvilli-like projections that form a „cuplike“ structure that surrounds leukocytes migrating through the endothelial cell body (28). In addition, endothelial ICAM-1 co-localises with the ring-like cluster of LFA-1 at the interface between the transmigrating leukocyte and endothelial junctions (27). The involvement of ICAM-2 in neutrophil diapedesis *in vivo* was demonstrated by intravital microscopy studies engaging blocking antibodies to ICAM-2 as well as with ICAM-2-deficient mice (30).

Endothelial adherens and tight junctions represent the major barrier for the transmigrating neutrophil in the paracellular path-

way (31). The major constituent of adherens junctions is VE-cadherin, which is the predominant gatekeeper for the passage of leukocytes. VE-cadherin disappears transiently from the junctions during transmigration (32), whereas blockade of VE-cadherin increases the rate of neutrophil extravasation *in vivo* (33). In contrast, the junctional adhesion molecules (JAM) and the JAM-related endothelial selective adhesion molecule (ESAM) are found in tight junctions of endothelial cells but also in circulating cells and belong to the immunoglobulin superfamily (34–36). A conserved motif in JAMs mediates their homophilic interaction (37, 38); however, JAMs have also the propensity to act as counter-receptors for leukocyte integrins: JAM-A, JAM-B, and JAM-C bind to LFA-1, VLA-4 and Mac-1, respectively (39–41). In addition, the interaction between JAM-C and JAM-B has been established (42, 43). Inhibition experiments as well as experiments with genetically modified mice have suggested a role of JAM-A and JAM-C in leukocyte recruitment *in vitro* and *in vivo*, especially at the step of diapedesis (44–48). The exact mechanism by which JAMs regulate leukocyte recruitment requires further investigation. This mechanism seems to be rather complex, as besides their homophilic and heterophilic interactions, JAMs can regulate the endothelial barrier. In particular, JAM-C and ESAM can disrupt VE-cadherin-mediated adherens junctions through the regulation of signaling pathways that involve small GTPases such as RAP1 or RhoA (49, 50), and these pathways may also participate in the modulation of leukocyte diapedesis.

A well established player in transmigration is platelet endothelial cell adhesion molecule-1 (PECAM-1), which is a member of the immunoglobulin superfamily and is expressed both on platelets and leukocytes as well as at the interendothelial junctions (51, 52), and can interact in a homophilic fashion to promote leukocyte transendothelial migration (53–55). PECAM-1 recycles in vesicular structures between the junctions and the subjunctional plasmalemma, and is thereby targeted to the area where leukocyte transmigration takes place (56). Besides its direct involvement in transmigration, PECAM-1 acts as a signalling receptor, as it contains two immunoreceptor tyrosine-based inhibitory motifs (57, 58) and its homophilic ligation also induces the upregulation of the laminin receptor $\alpha 6\beta 1$ -integrin on transmigrating neutrophils, thereby promoting the subsequent penetration of the basement membrane by these cells (59).

Regulation of integrin-mediated leukocyte adhesion

The major importance of integrin-mediated adhesion of leukocytes for the immune response is suggested by the immunodeficiency observed in the leukocyte adhesion deficiency syndrome (LAD I) in men lacking $\beta 2$ -integrins (18, 60), as well as by several studies engaging mice deficient in one or more leukocyte integrins (61–63). On the other hand, leukocyte integrins represent important therapeutic targets for disease, especially for autoimmune disorders. Blocking monoclonal antibodies against VLA-4 and LFA-1 are effective therapeutic strategies in multiple sclerosis and psoriasis, respectively (64, 65). Thus, understanding the

regulation of integrin-mediated leukocyte adhesion as well as the characterisation of exogenous and endogenous inhibitors of the leukocyte adhesion cascade is of major importance and will be the further focus of this review.

Leukocyte arrest on endothelial integrin ligands after variable periods of selectin-mediated rolling requires the proper activation of the integrins (10, 66–68). The two major mechanisms regulating the adhesive activity of integrins involve changes in their affinity and their valency. The regulation of integrin affinity for their respective ligands is mediated by conformational changes of the integrin subunits (10, 69, 70). An equilibrium of at least three different conformations of integrins exists on the cell surface: low-, intermediate- and high-affinity conformations (71–77). The transition from the low-affinity to the intermediate- and high-affinity conformation can be induced by separation of the cytoplasmic tails of the α - and β -subunits (76, 78). This separation is mediated by inside-out integrin signalling. Regulation of integrin valency involves changes of integrin distribution on the cell surface and clustering (10, 79).

Whereas both affinity and valency changes are involved in the regulation of leukocyte arrest (adhesion) on the endothelium (10, 66), regulation of affinity changes is the predominant mechanism for the induction of firm arrest/adhesion of leukocytes on vascular endothelium (3, 10, 80, 81). Inside-out signalling mediates the transition of the low-affinity to the high-affinity state. The contact of rolling leukocytes with the endothelium allows leukocytes to sense chemokines, presented on the apical endothelial cell membrane via heparan sulphate proteoglycans (17), which triggers integrin activation. In vitro systems resembling this process suggested that rapid triggering of integrin-dependent adhesion of leukocytes can be mediated by G-protein coupled receptors activated by immobilised chemokines (10, 82, 83), allowing leukocyte integrins to bind to their endothelial ligands (10, 66). The chemokine-induced signalling leading to integrin activation and subsequent firm leukocyte arrest happens in an immediate rather than in a stepwise successive manner (83). A key event transducing integrin-activating signals in leukocytes downstream of GPCR is the activation of PLC (84). PLC signalling subsequently induces activation of CALDAG-GEFI, a guanine exchange factor activating the small GTPase Rap1. GTP-bound (active) Rap1 rapidly stimulates integrin affinity and integrin-dependent adhesiveness through effector proteins such as RAPL and RIAM (10, 85–94). Moreover, RhoA is involved in mediating the chemokine-induced integrin activation in leukocytes (81, 95) (Fig. 1).

Additional pathways may be involved in the regulation of β 2-integrin activity in leukocytes. In this regard, the cytoplasmic tails of the α and β chains of the integrin can be phosphorylated during inside-out signalling activation (96–98). LFA-1 is constitutively phosphorylated on the α L-chain Ser-1140, which is important for the Rap1 pathway of integrin affinity regulation (96). In contrast, the phosphorylation of the β -chain of LFA-1 occurs upon cell stimulation, e.g. phosphorylation of T758 is induced by phorbol ester or downstream of T-cell receptor activation (99) and can mediate cytoskeletal interactions of the integrin with the multifunctional adaptor proteins of the 14–3–3 family, thereby modulating LFA-1-mediated cell adhesion and spreading (96, 100, 101). Further interactions between the integrin cytoplasmic

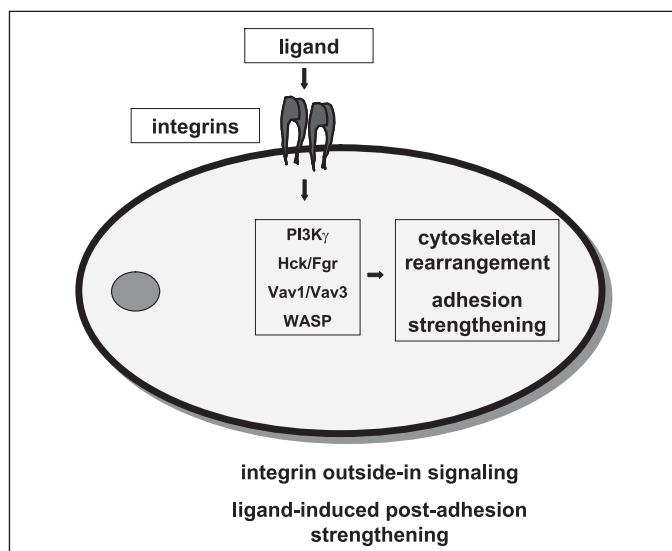


Figure 2: Integrin outside-in signalling. Binding of integrins to their respective ligands triggers intracellular signalling leading to a further increase of adhesivity. This so-called ligand-induced post-adhesion strengthening is relevant for the sustained adhesion of leukocytes onto the vascular endothelium and resistance to shear stress. PI3K γ , the src-like kinases Hck and Fgr, the GEF Vav1/Vav3 and WASP are implicated in the process of ligand-induced strengthening of adhesion.

tail and cytoskeletal proteins have been implicated in leukocyte adhesion. Talin is a central downstream effector in integrin activation. Talin associates with the cytoplasmic tail of the integrin and participates in a complex with the Rap1 effector, RIAM transmitting the integrin-activating Rap1 signals (102, 103). The binding of talin to the NPXY motif of the cytoplasmic tails of β -integrin subunits leads to separation of the cytoplasmic tails resulting in conformational rearrangements of integrin extracellular domains thereby inducing the intermediate or high integrin conformations (78, 104, 105) (Fig. 1).

Furthermore, selectin-mediated rolling of neutrophils can induce integrin activation. Binding of PSGL-1 with E-selectin induces the intermediate affinity conformation of LFA-1, which participates in both rolling and adhesive interactions (15). The PSGL-1-dependent activation of LFA-1 requires signaling pathways involving Fgr, DAP12, FcR γ and Syk kinase (16, 106).

Outside-in integrin signalling i.e. signaling following integrin ligation, is transduced through the cytoplasmic tails of the integrins and mediates the stabilization of initial adhesion. This pathway also designated as ligand-induced post-adhesion strengthening is relevant for the sustained adhesion of leukocytes onto the vascular endothelium and resistance to shear stress (107). Vav1 and Vav3 have been implicated in this process, since Vav1/Vav3-double deficient neutrophils displayed reduced sustained adhesion and spreading despite their normal arrest onto ICAM-1 under flow (108). Similarly, WASP-deficient neutrophils displayed normal rolling, integrin activation and firm arrest, but reduced resistance to detachment under flow (109). Furthermore, the src-like kinases Hck and Fgr are required for β 2-integrin-mediated outside-in signaling (107) (Fig. 2).

Inhibitors of the leukocyte adhesion cascade

Several exogenous microbial-derived inhibitors of the leukocyte adhesion cascade have been described. For example, the canine hookworm (*Ancylostoma caninum*)-derived neutrophil inhibitory factor (NIF) as well as the filamentous hemagglutinin (FHA) of *Bordetella pertussis* bind to Mac-1 integrin and potentially inhibit Mac-1-dependent leukocyte recruitment (110–112). In addition, *Staphylococcus aureus* contains several anti-adhesive and anti-migratory proteins that can interfere with multiple steps of host inflammatory cell recruitment, which have been recently reviewed (113). The staphylococcal superantigen-like protein-5 directly interacts with PSGL-1 thereby interfering with leukocyte rolling (114), whereas the staphylococcal extracellular adherence protein binds ICAM-1 resulting in potent inhibition of ICAM-1 interactions with β 2-integrins thereby preventing leukocyte recruitment *in vitro* and *in vivo* (115–118).

In contrast, less is known about endogenous inhibitors of the leukocyte adhesion cascade. Recent work from our lab has identified Developmental Endothelial Locus-1 (Del-1, *Edil3*) as a potent inhibitor of leukocyte recruitment. Del-1 is a secreted protein expressed by embryonic and some adult endothelial cells (119). Earlier studies implicated a role for Del-1 in angiogenesis (120–126); however, the Del-1-deficient mice are viable, fertile and display no obvious vascular defects (127). Del-1 consists of three epidermal growth factor repeats at its N-terminus and two discoidin I-like domains at its C-terminus (119). Interestingly, Del-1 is expressed in immunoprivileged tissues such as the brain and the eye as well in the lung vessels of adult mice (128). Del-1 is secreted by endothelial cells and most likely associates with the endothelial surface and matrix (125, 128, 129). Recent studies shed light to a novel function of Del-1 as an inhibitor of LFA-1-dependent inflammatory cell adhesion (128). Specifically, Del-1 was shown to associate with LFA-1, as murine neutrophils bound to Del-1 in a LFA-1-dependent manner. These findings were corroborated by experiments with LFA-1-transfected cells and solid-phase binding assays involving Del-1 and the I-Domain of LFA-1 locked in the open high-affinity conformation. Thus, Del-1 is a LFA-1-integrin ligand. However, in contrast to the classical LFA-1 ligand ICAM-1, Del-1 functioned to antagonise LFA-1-dependent adhesion to the endothelium (128). Immobilised Del-1 promoted only weak adhesion of leukocytes under physiologic flow conditions, whereas both soluble Del-1 as well as Del-1 co-immobilised with ICAM-1 inhibited the LFA-1-dependent adhesion of neutrophils to ICAM-1 under flow (128). Intravital microscopy studies *in vivo* revealed that increased numbers of leukocytes adhered to postcapillary venules of Del-1^{-/-} mice as compared to wild-type mice under both baseline conditions and upon tumor necrosis factor α stimulation (128). In addition, a significant reduction of the rolling velocity of leukocytes accompanied by an increase in the slow rolling fraction of leukocytes was observed in Del-1^{-/-} mice, which is consistent with the function of LFA-1 as mediator of slow rolling of leukocytes (16). Furthermore, enhanced neutrophil recruitment in LPS-induced lung inflammation was observed in Del-1-deficient mice, which was reversed in Del-1/LFA-1-double deficient mice, thereby demonstrating that Del-1 specifically interferes with LFA-1-dependent leukocyte recruitment (128).

Several possibilities could explain the anti-adhesive actions of Del-1 and are currently under investigation. First, secreted Del-1 may compete with ICAM-1 for binding to LFA-1. Second, Del-1 may act as an allosteric antagonist of LFA-1 inhibiting the conformational activation of LFA-1. However, we found no inhibitory effect of soluble Del-1 on LFA-1 affinity, as assessed by the induction of reporter epitopes on LFA-1, such as mAb24 epitope (Choi et al., unpublished data and [83]). Third, in contrast to other LFA-1 ligands, the binding of Del-1 to LFA-1 may be insufficient to induce post-adhesion strengthening or may negatively affect intracellular signaling pathways involved in the adhesion cascade. Fourth, Del-1 may also inhibit LFA-1-dependent leukocyte adhesion by affecting expression of ICAM-1. Indeed, we found increased ICAM-1 expression in Del-1^{-/-} endothelial cells and murine lung tissues under baseline conditions, however, this phenomenon was overcome upon pro-inflammatory stimulation (128). It is not clear to what degree the regulation of ICAM-1 expression by Del-1 contributes to the anti-inflammatory activities of Del-1. Although further investigations are required to clarify the mechanism of action of Del-1, Del-1 is an important endogenous inhibitor of inflammatory cell adhesion and homing.

Interestingly, the expression of Del-1 in endothelial cells and in mice was reduced upon proinflammatory stimulation (128), suggesting that inflammatory stimuli lead to a switch with increased expression of inflammation-promoting adhesion receptors (such as ICAM-1 or VCAM-1) and to reduced expression of inflammation inhibiting signals (such as Del-1). Extensive analysis by mass spectroscopy suggested the absence of soluble Del-1 from murine plasma (Choi et al., unpublished data). In addition, the discoidin domains of Del-1 protein contribute to its deposition in the extracellular matrix, a process that may involve interactions of Del-1 with glycosaminoglycans (129). Thus, Del-1 immobilised on the vascular endothelium most likely acts as a local and not systemic inhibitor of leukocyte adhesion, i.e. Del-1 acts in an autocrine / paracrine manner in the tissues that express it. However, one could envision that applying a form of soluble Del-1 appropriate to circulate in the plasma may be a novel therapeutic approach to inhibit inflammatory cell recruitment in disease.

Another endogenous inhibitor of leukocyte recruitment is galectin-1 (130, 131), that limits T-cell rolling and adhesion to activated endothelial cells under flow conditions. Galectin-1-deficient mice displayed enhanced homing of T lymphocytes to mesenteric lymph nodes and in a model of delayed-type hypersensitivity (130), as well as enhanced IL-1 β -induced leukocyte recruitment in the cremasteric circulation (131). Moreover, neuron-expressed soluble ICAM-5 can bind to LFA-1 and causes inhibition of T lymphocyte and microglia activation, in a manner opposite to the pro-inflammatory action of ICAM-1 (24, 132, 133). Interestingly, both Del-1 and ICAM-5 are highly expressed in the brain tissue and may thereby contribute to its immune privilege.

Conclusions

Despite the significant progress in the knowledge regarding the leukocyte adhesion cascade in the recent years, there are still sev-

eral gaps in our understanding. In particular, the relative importance of each of the adhesion receptors *in vivo* for tissue- and vascular bed-specific inflammatory cell recruitment needs to be established. In addition, identifying and understanding endogenous

inhibitors of the leukocyte adhesion cascade will not only increase the complexity of the cascade, but may also open the venue for designing novel therapeutic approaches in order to treat patients with autoimmune and inflammatory disorders.

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