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REVIEW

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Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules

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Abstract The emigration of leukocytes from the circulation is a critical step during immune surveillance and inflammatory reactions and is governed by a coordinated interplay involving a spectrum of adhesion and signal molecules. As the original multistep model of leukocyte trafficking undergoes continuous revision and refinement, the identification of additional molecules and the emergence of novel concepts for their intricately overlapping functions indicate that this process is still not completely understood. Early studies defining the multistep model described the rapid transition of selectin-mediated leukocyte rolling into integrin-dependent arrest followed by transendothelial diapedesis. It has become apparent that highly specialized chemoattractive cytokines termed chemokines and their heptahelical receptors are involved in the emigration of leukocytes not only by inducing chemotaxis but also by regulating integrins to trigger cell arrest in shear flow. In light of the enormously pleiotropic role of integrins and chemokines in vertebrate biology, this review summarizes and highlights selected aspects of currently evolving concepts refining the multistep model: (a) the differential activation of integrin avidity by chemokines and its implications, (b) the functional specialization of chemokines and their receptors in leukocyte recruitment, and (c) the multilayered molecular "zipper" controlling the completion of diapedesis at interendothelial junctions.

Keywords Integrins · Chemokines · Junctional adhesion molecule · Leukocyte · Emigration · Inflammation

Abbreviations *ARF:* ADP-ribosylation factor · *CCR:* CC chemokine receptor · *CXCR:* CXC chemokine receptor · *ERK:* Extracellular regulated kinase · *GEF:* Guanine exchange factor · *GRO:* Growth-related oncogene · *ICAM:* Intercellular adhesion molecule · *IL:* Interleukin · *JAM*: Junctional adhesion molecule · *LFA:* Lymphocyte function-associated antigen · *mAb:* Monoclonal antibody · *MCP:* Monocyte chemotactic protein · *MIP:* Macrophage inflammatory protein · *PECAM:* Platelet-endothelial cell adhesion molecule · *PH:* Pleckstrin homology · *PI3-K:* Phosphatidylinositol 3-OH kinase · *RANTES:* Regulated on activation normal T cell expressed and secreted · *SDF:* Stromal cell-derived factor · *TEM:* Transendothelial migration · *TNF:* Tumor necrosis factor · *VCAM:* Vascular cell adhesion molecule

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Introduction

The emigration of leukocytes from the circulation is controlled by a coordinated interplay of multiple signal and adhesion molecules, in particular selectins, chemoattractants, and integrins [1, 2]. The selectin family features molecules with a N-terminal domain homologous to lectins, which interact with sialylated carbohydrate determinants, for example, on mucinlike glycoproteins and mediate initial tethering to the vessel wall and rolling of leukocytes [1]. Chemoattractants are a diverse group of molecules which can activate heptahelical G protein cou-

pled receptors and induce the chemotaxis of leukocytes along a gradient [1]. Classical chemokines comprise formylated bacterial peptides (e.g., formyl-Met-Leu-Phe), complement components (e.g., C5a) or lipid mediators (e.g., platelet-activating factor) and attract most leukocyte subtypes. In addition, a polypeptide family of chemoattractive cytokines termed chemokines has been defined by sequence homology and has been classified according to the position of the N-terminal cysteine residues in CC chemokines, CXC chemokines, a CX_3C chemokine, and a C chemokine [2]. Consequently the G protein coupled receptors for chemokines have been grouped into CC chemokine receptors (CCR), CXC chemokine receptors (CXCR, or CX_3CR). More recently the nomenclature for the chemokine ligands (CC ligand and CXC ligand) has been modified to follow their receptors [3]. Integrins comprise a family of $\alpha\beta$ heterodimeric membrane proteins, which can be activated to bind to their immunoglobulin and matrix ligands by inside-out signaling after cellular stimulation [4, 5]. Mechanisms of their dynamic avidity regulation involve lateral clustering after release from cytoskeletal restraints, increases in affinity associated with conformational changes, and stabilizing postligand binding events such as cell spreading [5, 6, 7].

This three-step area code model initially proposed that selectin-carbohydrate, chemoattractant-receptor, and integrin-Ig ligand interactions act in sequence to achieve leukocyte emigration. The combinatorial use of such specifically distributed molecule pairs may determine the selectivity in the recruitment of distinct leukocyte subclasses. In particular, step 2 which involves a multitude of chemoattractant and chemokine receptor pairs was postulated to provide a sufficient choice of "digits" for all leukocyte subtypes. Recently it has been revealed that some elements can serve overlapping functions, for exampl, in integrin-mediated tethering and rolling, and that various integrins expressed on one leukocyte type or on different subclasses exhibit distinct patterns of regulation and function (part 1). Also, the apparent redundancy of chemokines may be explained by their differential immobilization and functional specialization (part 2), and novel junctional molecules involved in diapedesis have been identified (part 3). These developments are discussed as a part of the ongoing refinement of the multistep model and its plasticity in leukocyte emigration.

Tuning the instruments: differential regulation of integrins by chemokines to accomplish distinct steps of leukocyte recruitment

It has been convincingly demonstrated that chemoattractants induce the activation of leukocytic β_1 and β_2 integrins, which subsequently bind to endothelial Ig superfamily ligands or matrix proteins and facilitate the conversion of rolling into cellular firm adhesion [8]. The inhibition of leukocyte arrest and transendothelial migration (TEM) by the adenine dinucleotide phosphate ribosylating activity of pertussis toxin in an in vivo model has consistently demonstrated that a rapid G protein regulated activation event is involved in lymphocyte binding to high endothelial venules [9]. Similar to classical chemoattractants (e.g., C5a), the CXC chemokine interleukin (IL)-8 rapidly promotes adhesive interactions of the β_2 integrin Mac-1 and its ligands on endothelial cells, suggesting that it promotes leukocyte recruitment to sites of inflammation in vivo. These interactions occur independently of the Mac-1 surface density and thus require activation of Mac-1 avidity [10, 11]. Interestingly, the contribution of the β_2 integrins leukocyte function-associated antigen-1/$\alpha_L\beta_2$ (LFA-1) and Mac-1 to adhesion and/or TEM appeared to differ in resting and chemoattractant-stimulated neutrophils, i.e., while LFA-1 was crucially involved in both adhesion and TEM of resting cells, Mac-1 was additionally involved in formyl-Met-Leu-Phe stimulated cells [12]. On the other hand, the CC chemokine macrophage inflammatory protein (MIP) 1β has been shown to augment the adhesion of $CD8^+$ T cells to the $\alpha_4\beta_1$ integrin (very late activation antigen 4) ligand vascular cell adhesion molecule (VCAM) 1 in an elegant study [13] that also addressed the important role of chemokine immobilization. The identification of the heptahelical receptors CXCR1 and CXCR2 for IL-8 and its homologues [14, 15], as well as CCR1 and CCR2 for the CC chemokines MIP-1α/regulated on activation normal T cell expressed and secreted (RANTES; CC ligand 5) and monocyte chemotactic protein (MCP) 1 [16, 17], further confirmed that the functional activity of chemokines in leukocyte adhesion is mediated through well established G protein-coupled receptor pathways and initiated the surge of an ever growing family of chemokine receptors [3, 18, 19]. The astonishing redundancy of chemokines and their receptors is to some extent shared by integrins and their ligands. In addition to a leukocyte subtype specific expression of receptors, which may be responsible for triggering the selective recruitment to specific sites of inflammation, and emigrating leukocytes are exposed to multiple chemokines and integrin ligands at distinct steps of their extravasation. This implies that the specific mode and kinetics of integrin activation by chemokines may differ to allow their functional specialization during sequential events of the cascade, as illustrated in Fig. 1.

Chemokines differentially activate integrins with different β subunits on the same cell

A study with eosinophils, which express both β_1 and β_2 integrins has shown that chemoattractants and the chemokines RANTES and MCP-3 (acting via CCR3) induce a rapid and transient increase in the adhesiveness of $\alpha_4\beta_1$ for its ligand VCAM-1 [20]. This was associated with a reorganization of the actin cytoskeleton [20]. In contrast, the same agonists triggered a sustained activation of the β_2 integrin Mac-1 for its ligand intercellular adhesion molecule (ICAM) 1, which was mediated by a confor-

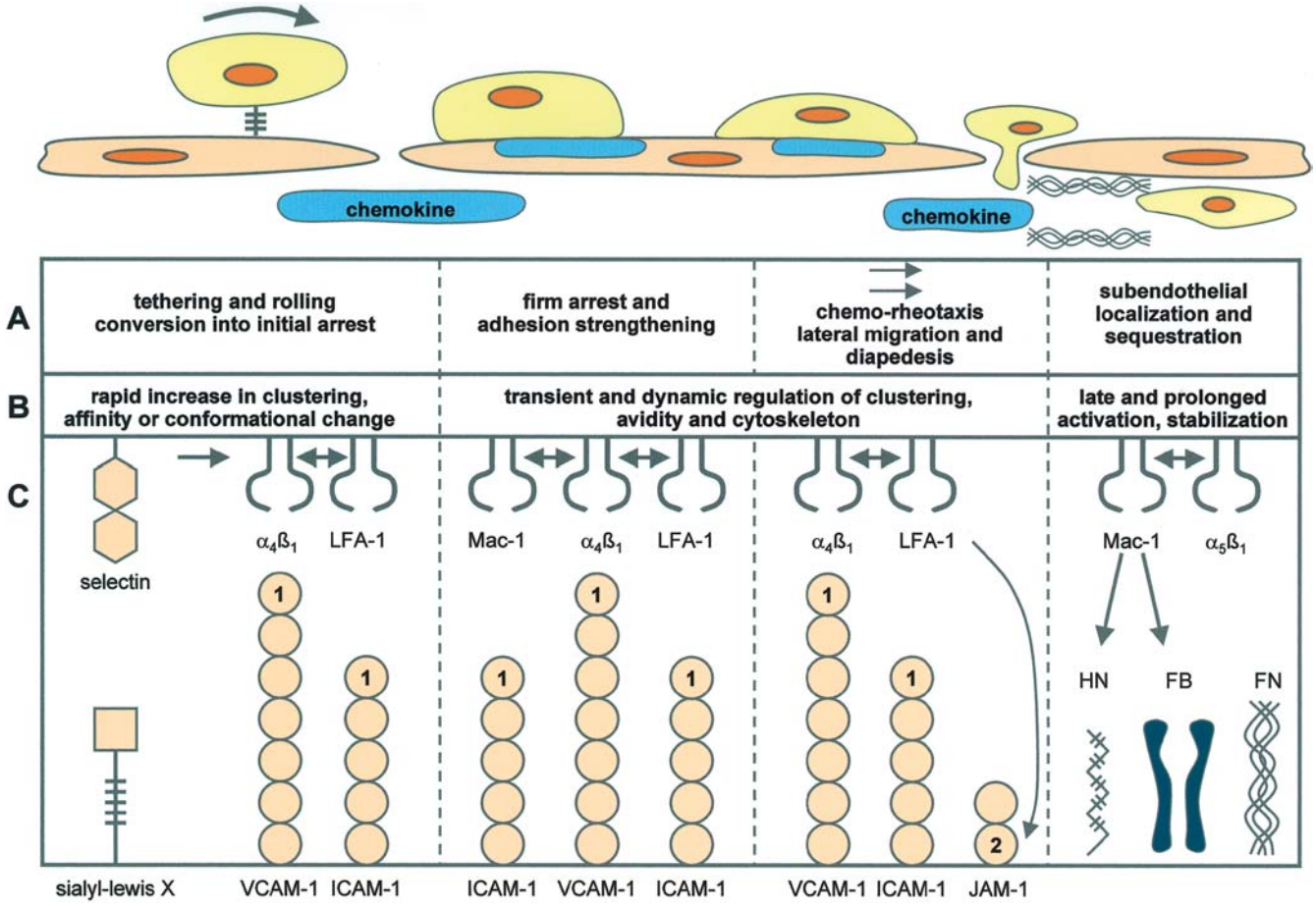

Fig. 1A–C Specialized functions of differentially regulated integrins during distinct steps of the sequential model for leukocyte extravasation. **A** The sequence of events during leukocyte emigration. In addition to selectin-carbohydrate interactions, both $\alpha_4\beta_1$ and LFA-1 can contribute to tethering, rolling, and conversion into arrest. Firm arrest and adhesions strengthening can be mediated by β_2 integrins or $\alpha_4\beta_1$, while the transient and dynamic regulation of $\alpha_4\beta_1$ and LFA-1 are important in lateral migration and diapedesis. Finally, persistent activation of $\alpha_5\beta_1$ and Mac-1 can support subendothelial localization. **B** The kinetic pattern and mode of regulation (e.g., increase in affinity or clustering) is attached to the different steps. **C** The interactions of integrins with their specific ligands. See text for further details. *ADP* Adenine dinucleotide phosphate; *ARF* 1/6 ADP-ribosylation factor 1/6; *CCL* CC chemokine ligand; *CCR* CC chemokine receptor; *CD* cluster of differentiation; *CS* chondroitin sulfate; *CXCL* CXC chemokine ligand; *CXCR* CXC chemokine receptor; *ELC* Epstein-Barr virus-induced receptor ligand chemokine (*MIP-3β*, *CCL19*); *ERK* extracellular regulated kinase; *FB* fibrinogen; *fMLP* formyl-Met-Leu-Phe; *FN* fibronectin; *GAG* glycosaminoglycan; *GEF* guanine exchange factor; *GRO*-α growth-related oncogene α; *HN* heparin; *HS* heparan sulfate; *ICAM*-1 intercellular adhesion molecule 1; *IFN*-γ interferon-γ; *Ig* immunoglobulin; *IL*-8 interleukin-8 (*CXCL8*); *IP*-10 interferon-γ inducible protein 10; *JAM*-1/2 junctional adhesion molecule 1/2; *LARC* liver- and activation-related chemokine (*MIP-3α*, CCL20); *LDL* low-density lipoprotein; *LFA*-1 lymphocyte function-associated antigen-1; *mAb* monoclonal antibody; *Mac*-1 αMβ2; *MCP*-1/3 monocyte chemotactic protein 1/3 (*CCL2/CCL7*); *MIP*-1β macrophage inflammatory protein-1β (*CCL3*); *MSGA* melanoma growth stimulatory activity; *PECAM*-1 platelet-endothelial cell adhesion molecule 1; *PH* pleckstrin homology; *PI3*-K phosphatidylinositol 3-OH kinase; *PKC* protein kinase C; *RANTES* regulated on activation normal T cell expressed and secreted (*CCL5*); *SDF*-1α stromal cell-derived factor-1α (*CXCL12*); *SHP*-2 src-homology 2-containing phosphatase-2; *SLC* secondary lymphoid tissue chemokine (*6-C-kine*, (*CCL21*); *TEM* transendothelial migration; *TNF*-α tumor necrosis factor α; *VCAM*-1 vascular cell adhesion molecule 1; *VE-cadherin* vascular endothelial cadherin

mational change reflecting an increase in affinity [21]. Further studies showed that eosinophils that we recovered from adhesion assays on a first integrin ligand underwent differential regulation on a distinct integrin ligand using a different chemotactic stimulus [20]. Alternatively, it cannot be ruled out that different subsets with distinct expression of receptors of specific coupling to integrin signaling cascades exist and partially account for these effects. This clearly indicates that integrins expressed on one cell population can be differentially activated. While the transient interaction of eosinophils through $\alpha_4\beta_1$ with endothelial ligands may support locomotion to intercellular junctions by reversible adhesive and detachment events, the prolonged activation of Mac-1 may cause a relative weak interaction, which may facilitate arrest without impairing diapedesis [20]. A cross-talk between integrins has been described for LFA-1 engagement, which decreased ligand binding of $\alpha_4\beta_1$, while $\alpha_4\beta_1$ engagement in turn initiated β_2 integrin-mediated adhesion [22, 23, 24], thus adding further

complexity. However, a transient mode of integrin regulation, for example, for $\alpha_4\beta_1$, can also directly result from stimulation with chemokines, while an eventual deactivation of Mac-1 may be triggered by signals from the endothelium. This provided the first evidence that β_1 and β_2 integrins expressed on the same cell can be differentially regulated by chemoattractants, and that the mode of activation is independent of the agonist but rather intrinsic to the integrin. Consistent with this, MCP-1 was found to selectively modulate the avidity of β_1 integrins for extracellular matrix proteins but not that of β_2 integrins for ICAM-1 in T lymphocytes, which do not express the myelomonocytic integrin Mac-1 [25]. Expression of the formyl-Met-Leu-Phe receptor or CXCR1 in lymphoid cells [26, 27] has also been reported to induce a differential regulation of integrin avidity with a transient activation of $\alpha_4\beta_1$ or $\alpha_4\beta_7$ and sustained increase in $\alpha_L\beta_2$ or $\alpha_v\beta_3$ avidity. As in eosinophils [20], this appeared to involve distinct cytoskeletal mechanisms and signal transduction elements. The unusual persistent activation of LFA-1 [26, 27] may be attributable to the ectopic environment and coupling to signaling cascades of an exogenously expressed receptor in lymphoid cells.

Chemokines dynamically trigger LFA-1 mediated arrest and transendothelial chemotaxis

Four chemokines have initially been shown to induce the LFA-1 mediated transient adhesion and rapid arrest of rolling lymphocytes on reconstituted endothelial substrates including immobilized chemokine or high endothelial venules under flow conditions [28, 29, 30]. Of these, stromal cell derived factor (SDF) 1α (CXC ligand 12), secondary lymphoid tissue chemokine (CC ligand 21), and Epstein-Barr virus induced receptor ligand chemokine (MIP-3β, CC ligand 19) but not MCP-1 or RANTES induced adhesion of most circulating lymphocytes, such as $CD4^+$ T cells, while liver- and activation-related chemokine (MIP-3α, CC ligand 20) triggered adhesion of memory but not naive $CD4^+$ T cells. This indicates that certain chemokines can trigger the arrest of lymphocyte subsets in flow via activation of LFA-1 avidity, which may allow them to control lymphocyte-endothelial cell recognition and recruitment in vivo. In contrast, RANTES has been shown to upregulate adhesion of $CD4^+$ T cells to extracellular matrix [31] and to trigger the arrest of Th1-type $CD4^+CD45RO^+$ memory T lymphocytes on activated endothelium in flow [32], events that are likely to be mediated by β_1 integrins. However, it has been revealed that some of the same chemokines, for example, SDF-1α and RANTES, fail to induce β_1 integrin-mediated adhesion of Th2-type T cells to extracellular matrix ligands, which may thereby limit recruitment to inflammatory sites characterized by thickening of the basement membrane or fibrosis [33]. The failure to demonstrate an upregulation of LFA-1 avidity with certain chemokines [25, 28] may thus be due to differences in the assay systems, chemokine receptor-specific uncoupling from LFA-1 activation, or leukocyte subset-specific differences in integrin activation or suppression. Since, for instance, in Th2 cells no obvious alteration in the signaling events has been identified [33], the exact mechanisms for this specificity are still only rudimentarily understood.

In further support of an immediate LFA-1 regulation, secondary lymphoid tissue chemokine, Epstein-Barr virus induced receptor ligand chemokine, or SDF-1α can induce both a high affinity state and lateral mobility of LFA-1 that together determine the arrest of circulating lymphocytes on ICAM-1 in flow [34]. This can be achieved by enhancing the frequency of ligand encounters via receptor clustering at low ligand density or increasing receptor affinity at high ligand densities. While inhibitors of phosphatidylinositol 3-OH kinase (PI3-K) or proteases, such as calpain, block LFA-1 mobility and clustering by preventing release from cytoskeletal restraints, the mechanisms of affinity changes remain to be elucidated. Following arrest the dynamic and transient LFA-1 regulation by chemokines in mononuclear cells is crucial for LFA-1 mediated diapedesis [35]. While MCP-1 induced LFA-1 dependent TEM of Jurkat transfectants coexpressing CCR2 and wild-type α_L, no TEM was observed with truncation mutants of the α_L cytoplasmic tail, which rendered LFA-1 either constitutively active or locked in a low avidity state that was not responsive to cell activation. Moreover, TEM was abolished by truncation of the β_2 cytoplasmic domain, indicating that TEM requires both α_L and β_2 cytoplasmic domains. MCP-1 induced TEM of mononuclear cells was also inhibited by a sustained extracellular activation of LFA-1 affinity. Dimeric soluble ICAM-1 also reduced transendothelial chemotaxis of mononuclear cells, implying that TEM involves at least transient changes in LFA-1 avidity, i.e., clustering or affinity [35].

Sequential regulation of integrins is dependent on the α subunit

As for LFA-1 a similar function in leukocyte recruitment to target tissues has been revealed for $\alpha_4\beta_1$, which promotes the conversion of initial rolling attachments to vessel wall ligands into firm arrest triggered by endothelial chemokines or chemoattractants [36, 37]. Immobilized chemokines can enhance arrest but also early integrin-mediated capture (tethering) of lymphocytes on inflamed endothelium. When presented in juxtaposition to the endothelial $\alpha_4\beta_1$ ligand VCAM-1, chemokines rapidly augment reversible lymphocyte tethering and rolling adhesions. Chemokines potentiate $\alpha_4\beta_1$ tethering within less than 0.1 s of contact through G_i protein signaling, the fastest inside-out integrin signaling event known to date [36]. Although $\alpha_4\beta_1$ affinity appears unaltered by chemokine signaling, subsecond $\alpha_4\beta_1$ clustering at the leukocyte-substrate contact zone results in enhanced leukocyte avidity to VCAM-1.

Leukocyte TEM may subsequently require an extension of the dynamic regulation of $\alpha_4\beta_1$ or other β_1 inte-

grins for endothelial and extracellular matrix ligands. Adhesion assays with monocytes on VCAM-1, fibronectin or its fragments revealed distinct patterns of kinetics for the regulation of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ avidity by CC chemokines (MIP-1α, RANTES, or MCP-1). Whereas chemokines induced an early activation and deactivation of $\alpha_4\beta_1$, the upregulation of $\alpha_5\beta_1$ avidity occurred late and was persistent [38]. Controlled detachment assays in shear flow confirmed that chemokines rapidly increased and subsequently reduced the adhesive strength of $\alpha_4\beta_1$ on VCAM-1 or the 40-kDa fragment of fibronectin, while inducing delayed and sustained adhesiveness of $\alpha_5\beta_1$ on a 120-kDa fibronectin fragment. In extension to the differential regulation of β_1 and β_2 integrins, these findings show that chemokines can differentially and selectively regulate the avidity of integrins sharing a common β subunit. Moreover, these data imply that the specific mode of activation is transmitted or determined by the cytoplasmic tails of α_4 and α_5, as previously suggested by their distinct functional effects [39]. The transient $\alpha_4\beta_1$ activation and deactivation may promote TEM of monocytes across VCAM-1 bearing barriers, while the delayed $\alpha_5\beta_1$ activation may mediate subsequent interactions with the basement membrane and localization of infiltrating leukocytes in the subendothelial extracellular matrix. This has also been suggested by a study with T cells in which CC chemokines, for example, RANTES, induced a prolonged binding to secreted extracellular matrix via β_1 integrins [31]. Interactions of $\alpha_4\beta_1$ with VCAM-1 or the 40-kDa fibronectin fragment support chemokine-induced random monocyte migration, which was optimal at intermediate site density. Chemokinesis on VCAM-1 appears to be associated with a transient $\alpha_4\beta_1$ regulation by chemokines, as locomotion rates were inversely correlated with the adhesive strength of $\alpha_4\beta_1$ to VCAM-1 [40]. Notably, locking $\alpha_4\beta_1$ in a high-avidity state, which cannot be modulated by chemokines suppressed random migration and transendothelial chemotaxis, particularly $\alpha_4\beta_1$ dependent TEM across VCAM-1 expressing endothelium [38, 40]. Induction of VCAM-1 expression by IL-4 improved lateral migration towards an MCP-1 gradient on endothelium and enhanced monocyte TEM by an $\alpha_4\beta_1$ mediated mechanism without affecting the time required for diapedesis per se [40]. Thus, transiently regulated interactions of $\alpha_4\beta_1$ with VCAM-1 can facilitate TEM by supporting lateral migration of attached monocytes along the endothelium. In conclusion, chemokines appear to regulate most if not all $\alpha_4\beta_1$ mediated steps in adhesive cascades that control leukocyte recruitment.

To further elucidate the molecular mechanisms responsible for a differential regulation via the α subunit of integrins, which share a common β subunit, the regulation of $\alpha_L\beta_2$ and β_2 integrin Mac-1 was investigated using cellular and integrin chimeras [41]. In parallel to the differential regulation of β_1 integrins, CC chemokines induced a sustained increase in monocyte adhesion to ICAM-1 that was mediated by Mac-1 but not LFA-1. However, the expression of an activation epitope revealed a rapid and transient upregulation of LFA-1 activity by MCP-1 in monocytes and Jurkat CCR2 transfectants, or by SDF-1α in Jurkat cells [41]. The expression of chimeras consisting of α_L and α_M cytoplasmic domain exchanges in α_L deficient Jurkat cells indicated that α cytoplasmic tails conferred the specific mode of regulation. Coexpressing α_M or chimeras in mutant Jurkat cells with a "gain of function" phenotype that resulted in a constitutively active LFA-1 showed that Mac-1 was inactive, while constitutive activity was mediated via the α_L cytoplasmic tail. This implied the existence of distinct signaling pathways for LFA-1 and Mac-1 [41]. Monocyte TEM in response to MCP-1 was dependent on LFA-1, while Mac-1 was involved only when its adhesive function was activated, revealing differential contributions of β_2 integrins. This was consistent with findings in neutrophils that LFA-1 is more important than Mac-1 in the inflammatory emigration of neutrophils [10], and that CXC chemokines induce a kinetically differential regulation of LFA-1 and Mac-1 [42]. Using ICAM-1 coated beads, it was shown that optimal rates of LFA-1 adhesion were transient and decreased within 1 min after chemokine stimulation, while Mac-1 adhesion continued to rise at later time points. Further differences were reflected by the topographical distribution of both integrins; whereas LFA-1 remained within lamellipodial regions, Mac-1 translocated to the cell uropod [42]. These data indicate that a specific regulation of β_2 integrin avidity by chemokines may be important for distinct functional contributions to leukocyte extravasation and may be triggered by distinct activation and signaling pathways transduced via the α subunit cytoplasmic domains.

Signal elements essential for integrin regulation: H-Ras and its effector kinases

There is a growing body of evidence that small GTPases of the Ras family are involved in the regulation of integrin avidity [3]. While R-Ras has been found to increase the avidity of β_1 integrins, which was associated with cell spreading [43], H-Ras suppressed β_1 and β_3 integrin activation via the Raf-1/extracellular regulated kinase (ERK) pathway and resulted in decreased integrin affinity [44]. These studies were largely restricted to malignant cell types or to integrins not expressed in their natural cellular context. Other studies in leukocytes stimulated by T-cell receptor engagement or IL-3 reveal that H-Ras may signal to activate the avidity of β_1 and β_2 integrins independently of the Raf-1/ERK pathway, implying a more complex role of H-Ras in integrin activation [45]. It has been shown that chemokines, for example, SDF-1α, can induce the activation of two downstream effectors of Ras, i.e., ERK and PI3-K [34, 46]. This is also reflected by a rapid increase in the phosphorylation of ERK and Akt, elicited by SDF-1α in Jurkat T cells. Expression of a dominant active form (D12) of H-Ras enhanced ERK phosphorylation, while dominant

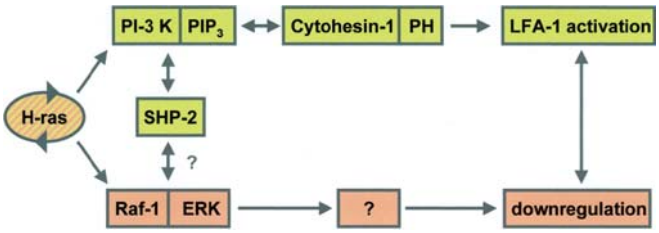

Fig. 2 Dual role of H-ras in the transient or cyclical regulation of LFA-1 avidity. While the H-Ras mediated activation of PI3-K can trigger membrane recruitment of cytohesin-1 via its PH domain and thereby induce LFA-1 activation in response to chemokines, H-Ras is also involved in a suppressive pathway mediated via the Raf-1/ERK pathway and yet unidentified effectors to downregulate LFA-1 activity. The phosphatase src homology 2 containing phosphatase 2 may be an element linking the two pathways. See text for further details (abbreviations: see Fig. 1)

negative N17 H-Ras only slightly impaired ERK activation but abrogated Akt phosphorylation in response to SDF-1α, indicating that its effect is due to inhibition of PI3-K.

SDF-1α triggered a transient regulation of adhesion mediated by LFA-1 and $\alpha_4\beta_1$, and a rapid increase in LFA-1 affinity for soluble ICAM-1, which was inhibited by D12 but not N17 H-Ras. Both D12 and N17 H-Ras abolished the regulation of LFA-1 but not $\alpha_4\beta_1$ and impaired LFA-1 but not $\alpha_4\beta_1$ dependent transmigration induced by SDF-1α. Inhibition of PI3-K blocked upregulation of LFA-1 mediated adhesion by SDF-1α, while inhibition of mitogen-activated extracellular signal regulated kinase activating kinase kinase impaired the subsequent down-regulation and blocking both pathways abrogated LFA-1 regulation [47]. Thus the inhibition of initial PI3-K activation by inactive H-Ras or sustained activation of an inhibitory ERK pathway by active H-Ras both prevail to abolish LFA-1 regulation and TEM induced by SDF-1α in leukocytes, establishing a complex and bimodal involvement of H-Ras (Fig. 2). Expression of the dominant inactive H-Ras strongly inhibited the SDF-1α induced Akt phosphorylation but not ERK phosphorylation. While the down-regulation of LFA-1 avidity appears to be mediated by the ERK pathway and possibly src homology 2 containing phosphatase 2, PI3-K activation was responsible for the rapid increase in avidity (C.W., unpublished data). In parallel with data using IL-3 [45], such differences may indicate that SDF-1α is an extremely potent stimulus for ERK phosphorylation, while PI3-K activation is more susceptible to dominant inactive H-Ras. Alternatively, chemokines may activate an upstream regulator of ERK that may bypass H-Ras. LFA-1 activation by chemokines has been shown to involve both an increase in lateral clustering and affinity changes [34]. While PI3-K appears to be crucial in mediating lateral mobility, it does not appear to play a direct role in inducing LFA-1 affinity, although lateral mobility may facilitate induction of high affinity. Since N17 H-Ras inhibited PI3-K activation but did not interfere with soluble ICAM-1 binding induced by SDF-1α [47], PI3-K does not appear to be involved in regulating LFA-1 affinity.

As a direct effector of G_i protein mediated chemokine signaling and H-Ras, PI3-K has been implicated in the cytoskeletal remodeling required for leukocyte spreading, polarization and chemotaxis during inflammation, and the inside-out signals converging with those of protein kinase C to activate integrin avidity [48, 49]. PI3-K can trigger LFA-1 mediated adhesion by inducing the membrane recruitment of cytohesin-1, which directly interacts with the β_2 subunit to increase LFA-1 avidity [50]. Since PI3-K may facilitate the activation of the Raf-1/ERK pathway [51], a sequential involvement of PI3-K and ERK may control the transient regulation of LFA-1 avidity by chemokines. The role of a cyclical activity of H-Ras in chemokine-induced leukocyte TEM extends results that the dynamic regulation of cdc42 is critical for monocyte chemotaxis by the development of actin-based filopodia [52]. In contrast, active H-Ras can cause sustained LFA-1 specific adhesion, which was mediated by PI3-K and triggered by immobilized or endogenous MIP-1α [53]. Since others have found that chemokines, such as SDF-1α, induce a transient increase in LFA-1 avidity [28, 34, 41], these differences may suggest cell- or chemokine-specific signaling pathways for integrin regulation. Unlike for LFA-1, dominant inactive or active H-Ras mutants did not affect $\alpha_4\beta_1$ regulation by SDF-1α in Jurkat cells. The lack of involvement of the ERK pathway or PI3-K in $\alpha_4\beta_1$ activation by SDF-1α supports results in myeloma cells [54] and confirms that the mechanisms and signal transduction involved in chemokine-induced activation of β_1 and β_2 integrins may differ [20]. The regulation of $\alpha_4\beta_1$ did not require PI3-K or ERK but rather actin cytoskeletal rearrangement may be due to a constitutive avidity of $\alpha_4\beta_1$ expressed on mononuclear cells. This implies that PI3-K and ERK are involved mainly in regulating integrins, which are expressed in a default low-affinity state such as LFA-1. On the other hand, the maintenance of high $\alpha_4\beta_1$ affinity mediating the chemokine-triggered arrest of T cells under flow has been shown to require the src kinase $p56^{lck}$ via adhesion strengthening [55].

Cytohesin-1 as an integral regulator of LFA-1 functions in response to chemokines

As pointed out, the cytoplasmic protein cytohesin-1 has been identified as a regulator of LFA-1 avidity. Cytohesin-1, which consists of an N-terminal coiled-coiled domain, a central Sec7 domain and a C-terminal pleckstrin homology (PH) domain, interacts with the β_2 cytoplasmic domain of LFA-1, and serves as a guanine exchange factor (GEF) for adenine dinucleotide phosphate ribosylation factor (ARF) GTPases, thereby increasing LFA-1 avidity [56, 57]. The functional role of cytohesin-1 in leukocyte adhesion on activated endothelium in flow and subsequent TEM in response to chemokines has now been described [58]. Overexpres-

sion of cytohesin-1 in leukocytes increased LFA-1 dependent arrest triggered by chemokines on activated endothelium in flow, while reducing the fraction of rolling cells. Conversely, arrest was impaired by a dominant negative PH domain construct of cytohesin-1, which prevents its membrane recruitment by PI3-K activation but not by a mutant deficient in GEF activity. This indicates an involvement of the PH domain in arrest, while GEF function is not required but plays an auxiliary role. Expression of these constructs or a β_2 mutant interrupting its interaction with cytohesin-1 demonstrated that shape change and transendothelial chemotaxis involve both LFA-1 avidity regulation and GEF activity. As a potential downstream target ARF6 but not ARF1 was found to participate in chemotaxis. Effects of cytohesin-1 and ARF6 mutants suggest that cytohesin-1 and ARF6 are intimately involved in the dynamic regulation of complex signaling pathways and cytoskeletal processes governing LFA-1 functions in leukocyte recruitment. Cytohesin-1 plays a multifunctional and integrative role in both conversion of rolling into firm arrest and TEM triggered by chemokines. Direct modulation of LFA-1 avidity or clustering contributes to arrest, while its GEF activity is crucial for shape change and TEM. In contrast, ARF6 is specifically involved in diapedesis. Identification of these proteins and their functional roles as important elements in the leukocyte cascade reveals that they may serve as checkpoints in inflammatory cell recruitment.

In conclusion, substantial advances have been achiev-ed in understanding the subtle choreography, the specific functional importance, and the molecular mechanisms driving the regulation of integrin avidity and cytoskeletal remodeling triggered by chemokines during leukocyte TEM. Similar to the variable requirement for chemokine-receptor combinations and signals, which can range from apparently redundant (e.g., the deficiency in CCR5 in some individuals) to essential (e.g., CXCR4, which is vital to embryogenesis), it is oversimplistic to assume that all specialized integrin functions driven by chemokines have consistent effects during leukocyte extravasation. Rather, some functions may be crucial and occur exclusively at some steps, while others may be overlapping or complementary, depending on the type of inflammatory scenario, leukocyte subpopulation and repertoire of chemokines, receptors and integrins expressed (Fig. 1). Finally, critical pieces to complete the picture still remain elusive. To name a few, the scaffolding and adapter molecules integrating multiple signaling pathways [59] and the interplay or cross-talk of such modular elements and effectors linking G protein coupled receptors to the subsecond activation of $\alpha_4\beta_1$ need to be identified. The distinct pathways transducing the signal to increase LFA-1 affinity (as opposed to avidity or clustering) in response to chemokines need to be defined.

Functional specialization of chemokines and their receptors for distinct steps of leukocyte recruitment: role of endothelial presentation and the division of labor

Leukocytes are confronted with a variety of apparently redundant chemokines that are presented in an inflammatory microenvironment. To further complicate the deceivingly simple task of guiding leukocytes across the endothelial barrier to their destinations, some chemokines have the potential to bind to more than one chemokine receptor expressed on the same cell. Furthermore, as a consequence of their relative promiscuity, most chemokine receptors bind more than one chemokine. Illuminating studies on the combinatorial control of leukocyte chemotaxis using an under-agarose assay have been shown that leukocytes encountering multiple chemotactic signals in complex spatial and temporal patterns can sort through and sequentially respond to a series of agonist gradients [60]. In this model migrating leukocytes can even integrate conflicting signals from competing chemoattractants and in recognition of their recent encounters display a bias for newly or subsequently encountered signals [61]. This provides an elegant basis for a multistep navigation of leukocyte migration through different chemokine signals. However, more physiological conditions of leukocyte recruitment in flow with different functional requirements, i.e., initial arrest, shape change, lateral migration, transendothelial diapedesis and orientation in the subendothelial matrix present a task even more challenging and complex to unravel.

Chemokine presentation may determine their sequential involvement in arrest or diapedesis

The functional specificity of chemokines has been associated with differences in their ability as cationic molecules to bind to cell surfaces via heparin-decorated proteoglycans or related glycoproteins [62, 63]. For example, adhesion of T lymphocytes to endothelium or subendothelial extracellular matrix can be triggered by MIP-1β or RANTES immobilized to endothelial heparan proteoglycans or CD44, whereas TEM of monocytes required a soluble gradient of endothelial-derived MCP-1 [13, 32, 64]. The CXC chemokine interferon-γ inducible protein 10 binds to a specific endothelial cell surface heparan sulfate site shared with platelet factor 4 [65]. The immobilization of chemokines to endothelial proteoglycans has been postulated to be particularly relevant under flow conditions, where they would be less susceptible to being washed away and, as opposed to soluble chemokines, may therefore efficiently recruit leukocytes [13, 62]. Soluble chemokines in the vascular lumen may be captured and immobilized by endothelial proteoglycans. As an additional mechanism of chemokine presentation, IL-8 has been shown to be abluminally internalized by endothelial cells, to be transported to the luminal surface in plasmalemmal vesicles and to be preferential-

ly located on microvillous projections where it is optimally positioned for leukocyte-endothelial contact [66]. Cytokine-activated endothelial cells synthesize and secrete both CC and CXC chemokines, i.e., MCP-1, IL-8, which acts via CXCR1 and CXCR2, and melanoma growth stimulatory activity α/growth-related oncogene (GRO) α, which acts via CXCR2 but not CXCR1 [14, 15, 16, 17, 67, 68]. It has been thought that CXCR2, a receptor for ELR-containing chemokines, is expressed predominantly on neutrophils. However, monocytes also express CXCR2, and GRO-α can stimulate changes in cytosolic Ca^{2+} and respiratory burst in monocytes [69, 70]. Moreover, surface-associated GRO on endothelium activated by modified low-density lipoprotein can promote monocyte adhesion [71]. Thus monocytes represent an excellent model to test the effect exerted by the presentation of endogenous endothelial chemokines on the functional specialization at distinct steps of inflammatory recruitment.

Differential immobilization and function of GRO-α and MCP-1 in monocyte recruitment

While GRO-α is immobilized on the surface of activated endothelia via binding to heparan sulfate proteoglycans, MCP-1 is secreted in a soluble form and thus subject to vascular flow. In accordance with these differences, the use of peptide antagonists revealed that GRO-α and CXCR2 are involved in conversion of rolling into firm arrest and adhesion strengthening of monocytes in physiological shear flow [72, 73, 74]. In contrast, MCP-1 and CCR2 were important in mediating subsequent shape change and TEM on stimulated endothelia. The latter was evident in flow but rarely in stasis and may thus require the establishment of a diffusible MCP-1 gradient or, as postulated for the paradigm of chemorheotaxis [75], the presence of shear forces. At the same time it has been shown that very high concentrations of MCP-1 and IL-8 added exogenously can trigger the firm arrest on a model substrate of resting but E-selectin transduced endothelium under shear flow [76]. This scenario may be relevant under circumstances where soluble chemokines can accumulate via transport to the luminal surface, as seen for MCP-1 in draining lymph nodes [77]. On the other hand, neutrophil arrest depends on the site density of the immobilized CXCR2 ligand IL-8, implying that a critical number of IL-8 molecules must be presented on the luminal surface to allow formation of high avidity integrin bonds sufficient for arrest [78]. Thus the differential modes of chemokine presentation on activated endothelium have been shown to determine the specific and hierarchical contribution of their receptors to the sequential induction of shear-resistant arrest vs. spreading and TEM of monocytes on inflamed endothelium [74]. In line with this, a compartment model for glomerular recruitment of monocytes revealed that CXCR2, surface immobilized GRO-α and fractalkine support arrest on glomerular endothelial cells, while MCP-1 triggers TEM towards the mesangium, thus establishing a combinatorial involvement of chemokines [79]. Although the precise nature of chemokine presentation has not been differentiated, a similar division of labor was confirmed by a study examining monocyte accumulation on atherosclerotic endothelium in ex vivo perfused carotid arteries of $apoE^{-/-}$ mice. Here, arrest of monocytes can almost exclusively be attributed to the closest murine ortholog to GRO-α, KC and its receptor CXCR2 but not to MCP-1 and CCR2, although both chemokines are displayed by luminal endothelium [80]. Due to the protection against atherosclerosis in MCP-1- or CCR2-deficient mice, as well as in chimeras harboring CXCR2-deficient monocytes [81, 82, 83], it can be concluded – by analogy to the in vitro results – that MCP-1 and CCR2 but not KC and CXCR2 must be involved in subsequent TEM. Since various CXCR2 ligands elicit differential responses in respiratory burst and chemoattraction, together these data suggest that not only chemokines but also their receptors can serve specialized functions, a hypothesis that has been explored and confirmed as outlined below.

A gradient of soluble MCP-1 is required for monocyte TEM [38, 64]. Since MCP-1 is not secreted in a polarized manner [64], other mechanisms are required for creating a gradient. As MCP-1 is not immobilized, this may enable MCP-1 secreted from the luminal side of vascular endothelium to be washed away under flow conditions, thus establishing and maintaining a soluble gradient. Indeed, polarized shape change and TEM of arrested monocytes rarely occurred under static conditions, and endogenous soluble MCP-1 was insufficient to mediate firm arrest on activated endothelium, as it may be washed away under flow conditions. Soluble eotaxin consistently augmented eosinophil binding in static assays; however, blocking its receptor CCR3 revealed only a slight contribution to firm eosinophil arrest on activated endothelium in flow [84]. This indicates a selective involvement of a diffusible MCP-1 gradient and its receptors, CCR2, in diapedesis of arrested monocytes.

Surface-bound chemokines and their receptors involved in shear-resistant leukocyte arrest

The lesser contribution of CXCR2 to monocyte diapedesis may reflect a solid immobilization of GRO-α on the luminal surface of endothelium, impairing the creation of an effective transendothelial gradient. Similarly, the CXC chemokines Mig and interferon-γ inducible protein 10 can be expressed and bound by endothelium stimulated with interferon-γ and tumor necrosis factor (TNF) α, and can induce the firm adhesion of T lymphocyte in shear flow via CXCR3. However, blocking CXCR3 did not reduce TEM [85], implying that other chemokine-receptor combinations may mediate diapedesis. The surface retention of chemokines, for example, GRO-α, may lead to higher occupancy of its receptor, which favors

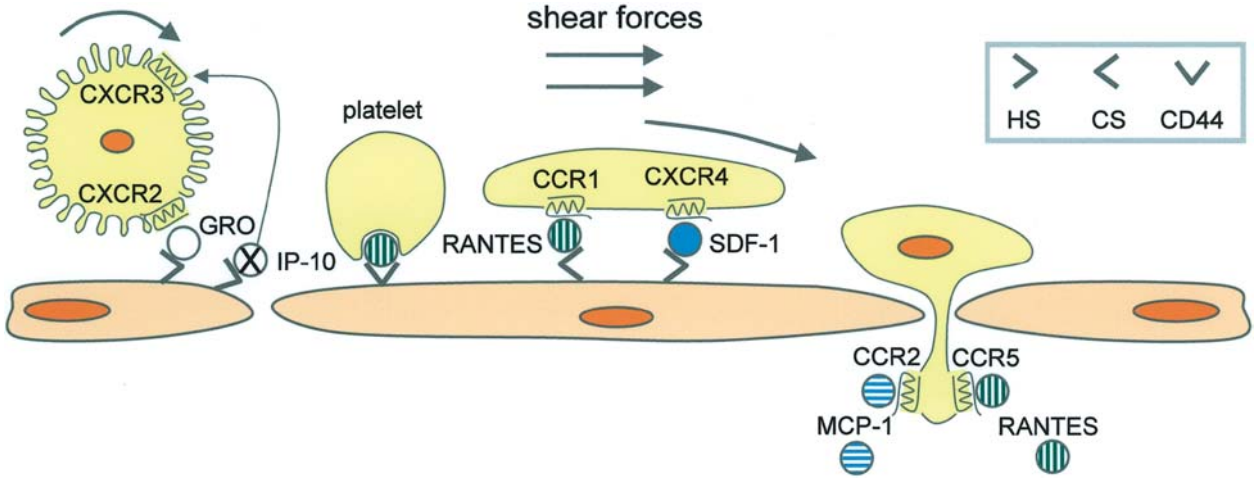

Fig. 3 Endothelial presentation and functional specialization of chemokines and their receptors for distinct steps of leukocyte recruitment: a division of labor. Chemokines immobilized to specific surface glycosaminoglycans (e.g., heparan sulfate or chondroitin sulfate), such as GRO-α, interferon-γ inducible protein 10, SDF-1α, or RANTES deposited by platelets, and their respective receptors (e.g., CXCR2–CXCR4, CCR1) are specialized in mediating shear-resistant arrest. Soluble gradients of chemokines, such as MCP-1, or immobilized SDF-1α under the influence of shear forces (chemorheotaxis) trigger subsequent transmigration. Moreover, chemokine receptors sharing the same ligand (e.g., RANTES) can be preferentially utilized to serve distinct functions, for example, CCR1 for arrest, CCR5 for spreading and transmigration. See text for details (abbreviations: see Fig. 1)

requirements for firm arrest rather than chemotactic responses [86]. Conversely, differential effects of GRO-α or MCP-1 may be due to uncoupling of chemokine binding from specific signaling pathways [87] or distinct requirements of proteoglycan binding for chemokine functions, i.e., triggering arrest vs. TEM [88]. The pluripotent role of chemokines is also exemplified by fractalkine, CX_3C chemokine, which consists of a chemokine domain situated on top of a mucinlike stalk [89]. While soluble forms of fractalkine induced leukocyte TEM, the membrane-bound form mediates adhesion of CX_3CR transfectants, in a process that is independent of G_i protein signaling or adhesion molecules [89, 90]. Mutational studies further showed that the CX_3C domain supports adhesion, while the mucinlike domain contributes to efficient presentation [91]. By analogy, CXCR2 on monocytes may directly mediate firm adhesion by interactions with GRO-α immobilized via heparan sulfates on activated endothelium. Together with findings that SDF-1α immobilized on endothelium can induce T cell arrest via CXCR4 [75], this implies that CXC chemokines and their receptors are well suited to induce mononuclear cell arrest on endothelium in flow. Differential presentation of chemokines in conjunction with a functional specialization of their receptors may provide a novel concept of their hierarchical participation in the complex process of leukocyte emigration (Fig. 3).

Intrinsic chemokine receptor specialization and the role of shear flow in transmigration

As an alternative explanation for specific effects during recruitment that are obscured by a multitude of potential chemokine-receptor combinations, the hypothesis that in addition to the impact of chemokine presentation, a functional specialization may be intrinsically determined by a receptor per se has been tested in a model in which RANTES immobilized on microvascular endothelium can trigger arrest of leukocytes [32, 91]. If correct, different RANTES receptors activated by the same chemokine should produce distinct functions. Using selective receptor antagonist, it was demonstrated that RANTES-induced monocyte arrest is mediated via its receptor CCR1 but not CCR5. Similarly, arrest of Th1 lymphocytes expressing both CCR1 and CCR5 was inhibited by blocking CCR1 but not CCR5 [32]. In contrast, CCR5 contributed to spreading of cells along the endothelium. However, in a transendothelial and transfilter chemotaxis assay, both CCR1 and CCR5 contributed to chemotaxis of monocytes to RANTES. These data reveal the selective use of chemokine receptors at different steps in the multistep process and may have dramatic implications in various disease states [32]. In a more pathophysiological model [92], RANTES secreted by thrombin-stimulated platelets was immobilized on the surface of inflamed microvascular or aortic endothelium and triggered shear-resistant monocyte arrest under flow conditions, as shown by inhibition with a RANTES receptor antagonist. Deposition of RANTES and its effects require endothelial activation, for example, by IL-1β. RANTES is also present on the luminal surface of carotid arteries of $apoE^{-/-}$ mice with early atherosclerotic lesions, after wire-induced injury or cytokine exposure [92]. In a mechanistic model of atherogenesis, monocyte adherence on endothelium covering such lesions has been studied in carotid arteries of these mice perfused ex vivo, showing that the accumulation of monocytes involved RANTES receptors. Hence deposition of RANTES by platelets triggers shear-resistant monocyte arrest on inflamed or early atherosclerotic endothelia. Delivery of

RANTES by platelets may epitomize a novel principle relevant to inflammatory or atherogenic monocyte recruitment from the circulation [92]. Although the basic residues critical for the binding of RANTES to proteoglycans with differential affinity have been identified [93], it remains to be conclusively demonstrated that modulation of surface binding in respective mutants affects their functions in arrest or TEM under flow and in vivo recruitment (Fig. 3).

Chemorheotaxis: transmigration triggered by immobilized chemokines in shear flow

Initial observations that monocyte TEM triggered by endogenous endothelial chemokines occurs more efficiently in flow than under static conditions [74] were reinforced by a report showing that shear potentiated TEM of neutrophils, which migrated with faster kinetics than under static conditions [94]. While these and other earlier studies support a transendothelial gradient of chemokines responsible for TEM, findings that RANTES added for apical binding on activated endothelium triggered CCR5-mediated spreading [32] in flow fostered theories that chemokines immobilized on the endothelial surface can also promote leukocyte TEM in shear flow. Recently an intriguing study has convincingly demonstrated that exogenous chemokines, namely SDF-1α, when immobilized on the apical surface of endothelial cells but not when used to pretreat lymphocytes in soluble form triggered robust TEM of lymphocytes in flow, which required physiological shear stress applied continuously to migrating lymphocytes [72]. This depended neither on endothelial permeability nor on the preexposure of endothelium to shear flow, which would redistribute apical chemokines permissive to TEM [75]. Both lymphocyte integrins $\alpha_4\beta_1$ and in particular LFA-1, an intact actin cytoskeleton and G_i protein mediated signaling, but not a chemotactic gradient, intracellular free calcium or intact PI3-K activity appeared to be mandatory for TEM. The inability of soluble chemokines to promote TEM implies that chemokines must be presented to migrating lymphocytes in the context of shear stress applied to the vessel wall, which may increase contact with the endothelium by deforming lymphocytes and thereby facilitating their exposure to displayed chemokines [94]. Torque forces generated by the fluid shear stress at this contact area may act as mechanical translators of signals transduced by apical chemokines. The strict shear stress dependence of chemokine-triggered TEM further suggests involvement of mechanosensitive regulatory elements on migrating lymphocytes. Thus lymphocyte TEM is promoted by fluid shear-induced mechanical signals coupled to G_i protein signals at apical endothelial zones, a phenomenon that has been coined with the term chemorheotaxis [75]. However, the enlightening conclusions from this study raise additional questions that will have to be clarified. Unlike lymphocytes, neutrophils and monocytes show substantial TEM without addition of apical chemokines and under static conditions (e.g., [32, 95]). This is likely to be due to a role of endogenous, apical and subluminal chemokines acting on myelomonocytic cells. Although the mechanisms of chemorheotaxis may be operative in neutrophils as well [75], combined and overlapping effects of endogenous endothelial chemokines and apically deposited chemokines derived from other sources, i.e., platelets, mononuclear or stromal cells remain to be elucidated in this context. The relevance of this concept should also be examined in physiological in vivo models with constant exposure to shear flow.

Passing the barrier: a multilayered "zipper" for leukocytes at endothelial junctions

The involvement of β_1 and β_2 integrins, in particular LFA-1 and its dynamic regulation in the process of TEM has been established in the reports discussed above, however, less is known about later steps controlling diapedesis through lateral adherens junctions between tightly apposed endothelial cells [96]. Although it has been suggested that TEM of neutrophils may involve a bypass of the junctional barrier and may occur at preexisting discontinuities at tricellular corners [97], leukocyte TEM has been associated with a reversible remodeling of the adherens junction between neighboring endothelial cells, suggestive of a junctional passage [98]. However, the exposure of endothelial cells to histamine, which increases permeability and clearly induces disorganization of adherens junctions does not augment or substitute for chemokine-triggered TEM of leukocytes [99]. Thus changes in endothelial permeability per se do not appear to be rate limiting for leukocyte TEM. Nevertheless, migrating leukocytes have been found to induce a delocalization of vascular endothelial cadherin from adherens junctions by yet unidentified mechanisms, thus disrupting a potential gatekeeping function of vascular endothelial cadherin in a confined region and allowing the leukocyte to transgress the opened gap [100]. While gap junctions, which constitute an exchange complex between adjacent cells for transport of ions and small molecules via transmembrane channels, have thus far not been implicated in the TEM of leukocytes, the strictly occluded barrier of the apical interendothelial tight junction must be parted by leukocytes extravasating different vascular beds [101]. The adhesive complex of tight junctions formed by tetraspan proteins (occludins or claudins) and junctional adhesion molecules (via association with cytoplasmic adaptors) appears to require an active and possibly reversible modification to be permissive for transendothelial diapedesis.

Role of immunoglobulin family members at the apical tight junction in transmigration

As one important component expressed at the apical regions of interendothelial junctions the Ig superfamily

member platelet-endothelial cell adhesion molecule (PECAM) 1 has been implicated in two distinct steps during the TEM of monocytes, neutrophils, and natural killer cells, as evident by inhibition with blocking antibodies or soluble PECAM-1 Fc chimeras [102, 103]. First, homophilic interactions of domains 1 and 2 of leukocyte PECAM-1 with their endothelial counterparts appear to contribute to diapedesis per se, since blockade of this step arrests leukocytes on the apical surface of the endothelium with pseudopods attempting to protrude into the junction. Second, the domain 6 of leukocyte PECAM-1 undergoes heterophilic interactions with yet unidentified binding partners in the basement membrane, since blocking this step retains leukocytes [104]. However, PECAM-1 deficient mice do not reveal a major defect in inflammatory disease models [105], indicating that a requirement of PECAM-1 is not obligatory for TEM. By analogy to the redundancy displayed in the cascade of TEM, this suggests a crucial involvement of other junctional molecules in the process of diapedesis. Junctional adhesion molecule (JAM) 1, an Ig superfamily member concentrated at endothelial junctions has been identified as engaging in homotypic interaction and to participate in the organization of the tight junctional complex and contact of neighboring cells [106, 107]. Inhibition with a mAb that does not affect homophilic interactions of JAM-1 as reflected by endothelial permeability revealed that JAM-1 is involved in TEM of monocytes and neutrophils [106, 108]. In contrast, murine JAM-1 antibodies that cause disruption of the endothelial integrity do not prevent leukocyte influx, which indirectly confirms the independence of permeability control and TEM [109]. On the other hand, the observation that a human JAM-1 mAb does not block monocyte TEM in flow may be due to the epitope specificity [110, 111]. The finding that the blocking mAb BV11 preferentially recognizes JAM dimers and their homophilic adhesive interactions indicates that these may be relevant to recruitment [112]. Since mouse monocytes may not express JAM-1 [106], these models indicate an effect on junctional rearrangement during TEM or more likely the existence of a JAM-1 ligand on leukocytes serving as a heterophilic interaction partner.

JAM-1 as a ligand for LFA-1 involved in transendothelial migration

Recent findings provide important pieces of information which help to resolve this conundrum. While the role of a dynamic regulation of LFA-1 avidity in governing the diapedesis step of leukocyte TEM has been documented [35], endothelial JAM-1 is now identified as a ligand for *trans*-interaction with LFA-1 and is crucially involved in leukocyte recruitment [113]. Under both static and flow conditions JAM-1 contributes to LFA-1 dependent TEM of T cells and neutrophils triggered by chemokines. Depending on its localization JAM-1 supported firm LFA-1 mediated arrest of T cells induced by chemokines, i.e., under conditions when its apical expression on endothelium is stimulated by a combination of TNF-α and interferon-γ [113]. The co-stimulation of endothelial cells with TNF-α and interferon-γ has been known to reduce junctional expression of PECAM-1 and JAM-1 by redistribution from interendothelial junctions, however, has yielded contradictory findings concerning TEM; in vivo recruitment is increased, TEM in stasis is reduced, and TEM in flow is unaltered [111, 114, 115]. Although this does not take into account contributions of other elements regulated by these cytokines or by shear flow, it has now been confirmed that a luminal expression of JAM-1 concomitant with redistribution is indeed permissive for LFA-1 dependent leukocyte recruitment. This implies that it may serve as a haptotactic gradient guiding attached leukocytes to diapedese. While the N-terminal domain of JAM-1 is responsible for its homophilic dimerization at interendothelial junctions, the membrane-proximal domain of JAM-1 supports its interaction with LFA-1. This structural duality may allow that homophilic interactions at interendothelial contacts may occur in parallel or in direct succession to heterophilic interactions of endothelial JAM-1 and LFA-1 on transmigrating leukocytes.

The homophilic association of JAM-1 relevant for dimerization has been suggested to involve the N-terminal region of JAM-1 [112]. A model based on the crystallographic structure of soluble recombinant JAM has proposed that U-shaped JAM dimers (interacting via such an N-terminal motif) are oriented in a *cis*-configuration on the cell surface and form a two-dimensional network by *trans*-interactions of N-terminal domains in a common central plane with dimers from an opposite cell surface [116]. Since LFA-1 binds to the membrane-proximal domain of JAM-1, which according to this model protrudes almost perpendicularly from cell surfaces, one may speculate that beyond a primary adhesive interaction with JAM-1, leukocytic LFA-1 may serve to intercept JAM-JAM interactions at junctions during TEM [113]. It is conceivable that homophilic interactions of JAM-1 via the N-terminal domain may still occur following the binding of LFA-1 to domain 2 to successively capture opposing JAM-1 dimers on the leukocyte and in turn to recruit endothelial JAM-1 as interaction partners for LFA-1. This scenario would be intriguing in the light of initial findings that the interaction of LFA-1 and JAM-1 was identified as occurring via their cytoplasmic domains, which may suggest a *cis*-interaction of LFA-1 and JAM-1 on leukocytes. This would parallel a report showing that PECAM-1, which also engages in homophilic associations, functions as a *cis*-interacting ligand for $\alpha_v\beta_3$ integrin [117]. The binding of LFA-1 on leukocytes to JAM-1 at interendothelial or interepithelial junctions may disrupt or intercalate homophilic junctional JAM-1 interactions [113], unlocking intercellular junctions and guiding leukocytes during TEM. Subsequently, homophilic interactions of JAM-1 may restore the junctional integrity. Thus, JAM-1 is ideally situated to provide a molecular “zipper” for TEM via a complex inter-

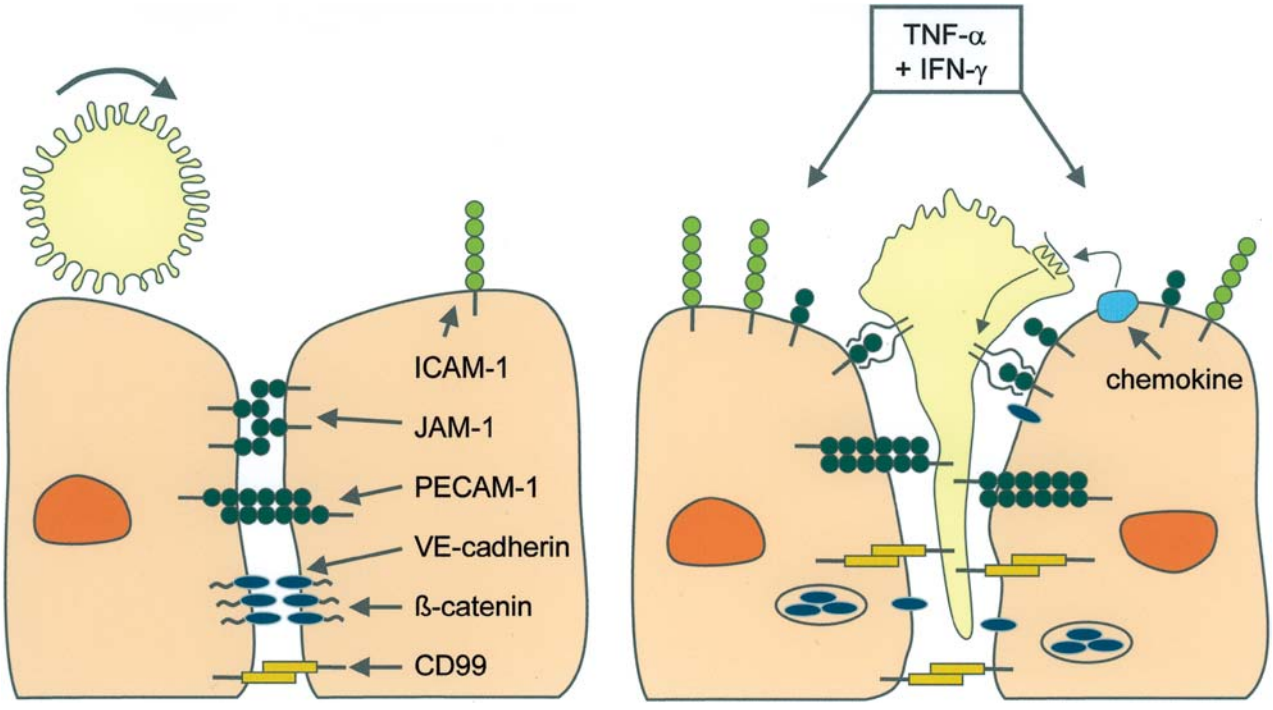

Fig. 4 Molecular mechanisms forming a multilayered "zipper" for leukocytes at endothelial junctions (modified from [120]). The combined stimulation of endothelial cells with inflammatory cytokines not only leads to the upregulation of Ig family members, such as ICAM-1 or VCAM-1, thereby promoting firm arrest of rolling leukocytes, but also induces a redistribution of JAM-1 from a junctional localization to the cell surface. Together with the dynamic regulation of LFA-1 binding to the domain 2 of JAM-1 by chemokines presented in the junctional vicinity, this may support the directional entry of leukocytes into the junction and subsequent diapedesis. Further migration to the abluminal side is mediated by sequential trans-homophilic interactions of PECAM-1 and CD99, which are less or little affected by stimulated redistribution, of JAM-2 and possibly of JAM-1. While these interactions may also assist in resealing the junction following leukocyte diapedesis, vascular endothelial cadherin is delocalized by transmigrating leukocytes from the adherens junction, creating a gap for subsequently transmigrating cells. See text for details (abbreviations: see Fig. 1)

play of its heterophilic and homophilic interactions. Future studies must to address the intricate cross-talk of these JAM-1 interactions in leukocyte TEM, the role of putative signaling pathways via the cytoplasmic domain of JAM-1, and other heterophilic interactions partners.

A multilayered molecular "zipper" for transendothelial migration

As an additional JAM family member engaging in homotypic interactions, murine JAM-2 (human JAM-3) has recently been implicated in the control of leukocyte TEM [118]. The endothelial expression of JAM-2 not only increased the permeability of monolayers but also promoted the migration of human leukocytes, which also express JAM-2 [118]. In contrast, a blocking mAb or soluble JAM-2 inhibited leukocyte TEM across human umbilical vein endothelial cells. Thus the role of JAM-2 in TEM may involve both an interplay with other JAM family members expressed in the vicinity, as well as a signaling function of JAM-2 due to homotypic interactions.

A multistep cascade involving sequential molecular interactions between emigrating leukocytes and endothelial junctions has been further supported by a report showing that CD99, a molecule which is expressed on most hematopoietic cells and concentrated at interendothelial contacts is essential for a late step in monocyte TEM [119]. This presumably involves homotypic interactions of CD99 on leukocytes and endothelial cells. Whereas blocking PECAM-1 arrests leukocytes on the apical surface of endothelium, blocking CD99 arrests monocytes distal to the PECAM-1 dependent step at a point where they are partially through the junction. This possibly involves blocking the uropod, the tail of the migrating cell and a membrane region enriched in adhesion molecules [120]. Thus, emigrating monocytes first use homophilic PECAM-1 interactions to penetrate the apical entry of the junction and initiate diapedesis. Homophilic CD99 interactions then allow the invading leukocytes to transmigrate through clefts in the endothelial wall and complete diapedesis. Since the expression and distribution of CD99 at interendothelial borders was not affected by inflammatory conditions this mechanism may form a basic housekeeping element for leukocyte TEM. Since CD99 regulates LFA-1 expression and affinity via an unknown signal-transduction mechanism [121], CD99 interactions may indirectly influence leukocyte TEM, possibly by regulating the function of integrins in the uropod. Thus diapedesis is sequentially regulated by two distinct molecules, i.e., PECAM-1 and

CD99. It remains to be determined at, which point of this sequence the postarrest involvement of JAM-1 and its engagement with LFA-1 as well as a intercellular function of JAM-2 participate in diapedesis. It is also unclear whether these constitute cooperative or alternative pathways, given the redundancy of PECAM-1 illustrated in deficient mice [105].

Taken together, compelling evidence has been provided for a multilayered molecular "zipper" enabling effective diapedesis of leukocytes without a detrimental leakage of the vascular endothelium (Fig. 4). It will be essential to further dissect and confirm the mechanisms of this multistep cascade for TEM in relevant in vivo models, for example, by employing intravital microscopy. In addition to the role of a dynamic integrin regulation by chemokines, the impact of chemokine immobilization, the functional specialization of their receptors and shear forces, evolving concepts epitomize the puzzling complexity in the control of leukocyte TEM. The considerable abundance of factors determining the specificity of leukocyte recruitment harbors an enormous potential for the identification of selective therapeutic targets and the development of antagonists for the treatment of inflammatory or cardiovascular diseases characterized by an inappropriate excess of leukocyte recruitment [122, 123].

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