

Arrest Chemokines

KLAUS LEY

Cardiovascular Research Center and Department of Biomedical Engineering,
 University of Virginia Health System, Charlottesville, VA, USA

ABSTRACT

In most organs, leukocyte attachment to the endothelium of blood vessels requires capture and rolling before firm adhesion is initiated by integrin activation and/or redistribution, which can be initiated by immobilized chemokines binding their cognate receptors on rolling cells. Such arrest chemokines are present on the endothelial surface under physiologic or pathologic conditions, necessary, and sufficient to trigger arrest. Although many chemokines can be immobilized and cause arrest of rolling cells in flow chambers, only four have so far been shown to function as arrest chemokines under physiologic conditions, although the actual number could be much higher. Secondary lymphoid tissue chemokine (SLC) (CCL21) on high endothelial venules triggers arrest of rolling lymphocytes, and keratinocyte-derived chemokine (KC) (mouse Gro- α , CXCL1), monocyte chemoattractant protein-1 (MCP-1) (CCL2), and regulated on activation, normal T cell exposed and secreted (RANTES) (CCL5) trigger arrest of rolling monocytes. Remarkably, no arrest chemokine for neutrophils under inflammatory conditions has been found so far. *Microcirculation* (2003) **10**, 289–295. doi:10.1038/sj.mn.7800194

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INTRODUCTION

Targeted delivery of leukocytes (e.g., neutrophils to sites of inflammation, lymphocytes to secondary lymphatic organs, eosinophils to inflamed bronchial tissue, or monocytes to atherosclerotic lesions) requires their rapid and selective activation while in the blood microvessels. This process coincides with their arrest from a rolling to a firmly adherent state in postcapillary venules, high endothelial venules of lymphatic organs, or atherosclerotic arteries. While the need for activation has long been recognized, soluble chemoattractants were initially thought to diffuse through the tissue and into the blood microvessels. Exogenously applied chemokines can trigger rapid arrest of rolling neutrophils (4,28,48), but this mode of application does not realistically mimic the situation during inflammation. With exogenous application, it is not clear that the dose applied is physiologically rel-

evant, and there is no control of the amount of surface-bound versus soluble chemokine. When it was shown that certain chemokines can be deposited on the surface of endothelial cells (41), produced by endothelial cells (19), or transported through endothelial cells to be presented in the lumen (32), these chemokines became the prime candidates for triggering arrest of neutrophils and other white blood cells.

DEFINITION OF ARREST CHEMOKINES

A concise definition of an arrest chemokine would have to include the following: an arrest chemokine is expressed or presented on the luminal surface of a micro- or macrovessel; blocking or removing this chemokine or its receptor blocks the arrest function; and adding the chemokine back to the endothelium restores the arrest function. The first report of a true arrest chemokine was provided by Gerald Nash's laboratory in 1995. They showed that isolated human neutrophils rolled and arrested on cultured human umbilical vein endothelial cells (HUVECs) exposed to hypoxia and reoxygenation and IL-8 (CXCL8) was required to trigger the arrest function (39). Although the reconstitution assay was not completed in this study, other published work suggested that endothelial-presented IL-8 would be effective (19). Nash's early work identified at least two modes of action of arrest chemokines. Leukocyte adhesion may either follow directly after initial contact

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For reprints of this article, contact Dr. Klaus Ley, University of Virginia Health System, Cardiovascular Research Center, MR5 Building - Rm. 1013, P.O. Box 801394, Charlottesville, VA 22908-1394, USA. For express mail, send to MR5 Building, 415 Lane Road, Charlottesville, VA 22903, USA; e-mail: klausley@virginia.edu

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with the endothelium or may require a distinguishable period of rolling (40).

MECHANISMS OF ARREST

Most known mechanisms of arrest require integrin activation and/or clustering on the cell surface. *In vitro*, arrest of rolling cells has been demonstrated through α_4 integrins (23), $\alpha_L\beta_2$ (LFA-1) (27), and $\alpha_M\beta_2$ (Mac-1) (9), depending on the cell types involved. Of the few arrest chemokines identified *in vivo* (see Table 2), lymphocytes in high endothelial venules use LFA-1 (44) and monocytes in atherosclerotic arteries use $\alpha_4\beta_1$ for arrest (20).

In vivo experiments have provided only circumstantial evidence that the integrin in question acquires the activated conformation. More direct observations have been possible in flow chambers *in vitro*. Low doses of soluble IL-8 in combination with L-selectin cross-linking potentiated neutrophil arrest on L-cells co-transfected with E-selectin and intercellular adhesion molecule-1 (ICAM)-1 under flow, and this arrest was only blocked when both LFA-1 and Mac-1 were blocked (16). Adhesion of human neutrophils to ICAM-1 coated beads in response to soluble Gro- α (CXCL1) (through CXCR2) or soluble IL-8 (through CXCR1) was LFA-1 dependent, with optimal adhesion at 1 min after stimulation (42). Mac-1-dependent adhesion was more prolonged, and beads bound to Mac-1 were translocated to the uropod of the neutrophils within 4 min (42). Shear-resistant binding of human neutrophils to ICAM-1 coated beads, a ligand for LFA-1 and Mac-1, was triggered by IL-8 (30). Similar concentrations of IL-8 induced neutrophil binding to beads and induction of the epitope recognized by mAb 327C; a reporter of the active conformation of the β_2 integrin I-like domain. This was accompanied by redistribution of LFA-1 into membrane patches, suggesting that both integrin affinity modulation and redistribution are important for neutrophil arrest under flow. A report from Steven Rosen's group showed that immobilized SLC (CCL21) induced β_2 integrin-dependent arrest of naïve T cells on ICAM-1 (46). Soluble secondary lymphoid tissue chemokine (SLC) or stromal cell-derived factor-1 (SDF-1) did not induce expression of an activation epitope on β_2 integrins detected by mAb24 (46). However, interpretation of this data is complicated because mAb 24 binds to a divalent cation- and ligand-dependent epitope and does not directly recognize the ligand-binding site of β_2 integrins. Constantin and colleagues reported that chemokine-induced adhesion of naïve mouse lymphocytes to immobilized ICAM-1 required redistribution of LFA-1 at low to intermediate site densities of ICAM-1

(7). This was inhibited by blocking PI3-kinase or calcium-dependent intracellular proteases. At very high site densities of ICAM-1, the requirement for LFA-1 redistribution was relaxed, but a transient affinity increase of LFA-1 was inferred from binding of soluble ICAM-1 to the lymphocytes. These results must be interpreted with caution, because the binding assay was performed at 37 °C, a temperature permissive for internalization of receptors and ligands. In this study, most experiments were not done in flow chambers, so lymphocyte arrest or a possible contribution of ligation of selectin ligands could not be demonstrated. Taking together the results from all these studies, it is likely that both affinity upregulation and surface redistribution of β_2 integrins contribute to chemokine-induced arrest, but conclusive experiments have not been presented.

Arrest of isolated naïve T cells to ICAM-1 and peripheral node addressin (PNA), a mixture of molecules with ligand activity for L-selectin) immobilized on a plastic surface occurs very rapidly (within less than 1 s) when the chemokines SDF-1 (CXCL12), SLC (CCL21), ELC (CCL19), or macrophage inflammatory protein (MIP)-3 α (CCL20) are co-immobilized (2) (Table 1). MIP-3 α has also been shown to support rapid arrest of memory T cells on dermal microvascular endothelial cells (11). However, neutrophil arrest on a surface containing ICAM-1, P-selectin, and IL-8 (10) or on platelet monolayers (9) requires a longer rolling interaction, and neutrophils rolling on activated endothelium *in vivo* typically require several minutes of rolling before arrest occurs (25). Of note, the putative arrest chemokines mediating neutrophil arrest *in vivo* are not known. Although *in vitro* evidence shows that IL-8 can act as an arrest chemokine for human neutrophils (10), mice lacking CXCR2, the receptor most closely related to the human IL-8 receptor, CXCR1, show normal numbers of arresting neutrophils in postcapillary venules of the TNF- α treated cremaster muscle (Dunne, Forlow, Ley, unpublished).

Immobilized chemokines including SDF-1 promote capture, rolling, and arrest through $\alpha_4\beta_1$ integrin (17). This enhanced capture occurs within 0.1 s of contact and requires G α_i -dependent signaling and $\alpha_4\beta_1$ integrin clustering. SDF-1 was obligatory for successful arrest, but activation of $\alpha_4\beta_1$ could not be demonstrated. Immobilized SDF-1 also promotes arrest of CD34⁺ bone marrow-derived hematopoietic progenitor cells on ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and tumor necrosis factor- α (TNF- α) activated endothelial cells (37). Interestingly, both soluble and surface-bound SDF-1 in-

Table 1. Arrest chemokines *in vitro*

Arrest chemokine	Chemokine receptor	Leukocyte type	Surface	Reference
SLC (CCL21), ELC (CCL19)	CCR7	Naïve T lymphocyte	L-selectin ligands (PNAd) and ICAM-1	(2,46)
RANTES (CCL5)	CCR1	Human monocyte, human T cell	TNF- α activated HUVEC	(50)
GRO- α (CXCL1)	CXCR2	Human neutrophil, monocyte	ICAM-1 beads, TNF- α activated HUVEC	(42)
IL-8 (CXCL8)	CXCR1	Human neutrophils, transfected Jurkat cells, pre-B cells, human monocytes	HUVEC after hypoxia-reoxygenation, VCAM-1, TNF- α activated HUVECs	(1,14,31)
Eotaxin (CCL11)	CCR3	Eosinophils	TNF- α and IFN- γ activated HUVEC	(8,24)
IP-10 (CXCL10), Mig (CXCL9)	CXCR3	IL2-treated T cells	TNF- α and IFN- γ activated HUVEC	(38)
SDF-1 (CXCL12)	CXCR4	Naïve CD4+ T cells, monocytes, CD34+ hematopoietic progenitor cells	P-selectin and ICAM-1 VCAM-1	(2,17,37) (5)
MIP-3 α (CCL20)	CCR6	Human blood lymphocytes	P-selectin and ICAM-1 Dermal microvascular endothelial cells	(2) (11)
MIP-1 α (CCL3)	CCR1	Transfected Jurkat cells, mouse pre-B cells	VCAM-1	(1)
Fractalkine (CX3CL1)	CX3CR1	Human monocytes, T cells, NK cells	Fractalkine, TNF- α activated HUVEC	(33)

duces arrest of human peripheral blood-derived lymphocytes to TNF- α activated HUVECs, but only surface bound and nonsoluble SDF-1 also promotes transmigration (6).

CCR3 ligation promotes attachment of eosinophils to ICAM-1 and VCAM-1, because a CCR3 antibody reduces eosinophil arrest in response to supernatant derived from cultured HUVECs stimulated with TNF- α and interferon- γ (IFN- γ) (24). Blocking CXCR1 or biosynthesis of platelet-activating factor had no effect on eosinophil accumulation on activated HUVECs (24). Interestingly, ligation of CCR3

by eotaxin (CCL11), eotaxin-2 (CCL24), or regulated on activation, normal T cell expressed and secreted (RANTES) (CCL5) on adherent eosinophils promotes their rapid detachment from VCAM-1 in a flow chamber assay (45). Simultaneously, adhesion to denatured bovine serum albumin, a ligand for Mac-1, was enhanced, suggesting that eotaxin-2 or other CCR3 ligands may shift integrin usage from $\alpha_4\beta_1$ to $\alpha_M\beta_2$.

IL-2 stimulated T cells express CXCR3, and the CXCR3 ligands γ -interferon-inducible protein (IP)-10 and Mig induce rapid arrest of these cells (38). As

Table 2. Arrest chemokines *in vivo*

Arrest chemokine	Chemokine receptor	Leukocyte type	Blood vessel type	Reference
SLC (CCL21)	CCR7	Mouse T lymphocytes	Mouse lymph node high endothelial venule	(44)
RANTES (CCL5)	CCR1 (CCR3, 5 not excluded)	Human monocytes	Atherosclerotic mouse carotid artery	(47)
KC (mouse GRO- α , CXCL1)	CXCR2	Mouse monocytes	Atherosclerotic mouse carotid artery	(20)
MCP-1 (CCL2)	CCR2	Mouse monocytes	High endothelial venules	(36)

IP-10 (CXCL10) and Mig (CXCL9) are expressed by HUVECs activated with TNF- α and IFN- γ , they are candidate arrest chemokines for activated T cells. *In vivo*, CD8⁺ T cells roll in cremaster venules after stimulation with TNF- α and IFN- γ through an α_4 integrin-dependent mechanism (43), but it is not known which chemokines mediate their arrest.

A completely different arrest mechanism can be mediated by fractalkine, a tethered chemokine that binds to CX3CR1. Monocyte, T cell, and natural killer cell arrest without integrin involvement was demonstrated on fractalkine fusion protein, fractalkine-transfected cells, and TNF- α activated HUVECs (12). Arrest was not blocked by pertussis toxin, an inhibitor of G α_i -mediated signaling, chelation of divalent cations, or antibodies to integrins. The importance of this pathway of arrest *in vivo* is unknown. Mice lacking the fractalkine receptor CX3CR1 show no severe spontaneous phenotype (22) and support enhanced survival of cardiac allografts (18), but it is not known whether this is related to the putative arrest function of fractalkine.

DIFFERENTIAL ACTIVATION OF INTEGRINS

An early report from Eugene Butcher's laboratory showed that CXCR1 and CCR1 transfected into murine pre-B cells or human Jurkat cells promoted the arrest of these cells under flow in response to IL-8 (CXCL8) and MIP-1 α (CCL3), respectively (1). This required a high local concentration of the respective chemokine. The authors concluded that there is a quantitative difference between the arrest and the chemoattractant functions of chemokines. Chemokine receptors as well as receptors for other chemoattractants like C5a can trigger arrest, suggesting that in the cells tested the signaling machinery necessary for induction of arrest is present and also coupled to the receptors. However, this view based on experiments with transfectants was later modified, because preferential integrin activation was observed in natural leukocyte subsets.

Weber and colleagues (49) showed that functional activation of $\alpha_4\beta_1$ by MIP-1 α or RANTES occurred much more rapidly than activation of $\alpha_5\beta_1$, suggesting that $\alpha_4\beta_1$ may be involved in arrest and $\alpha_5\beta_1$ in subsequent migration. Indeed, arrest of monocytes on atherosclerotic mouse carotid artery walls was later shown to be $\alpha_4\beta_1$ -dependent (20). Activation of cultured HUVECs with TNF- α induces expression of both Gro- α and monocyte chemoattractant protein-1 (MCP-1), but only Gro- α is efficiently immobilized on the endothelial cell surface and promotes ar-

rest of rolling monocytes whereas MCP-1 is secreted in a soluble form (51). MCP-1 decreased rather than increased monocyte adhesion to VCAM-1 (49) or had no effect (3) although exposure to soluble MCP-1 increased monocyte arrest on HUVECs transfected with VCAM-1 and E-selectin (14). Blocking MCP-1 or CCR2 had no effect on monocyte arrest on atherosclerotic carotid arteries *in vivo* (20). Taken together, these data suggest that in large arteries, MCP-1 may have a function downstream from arrest; for example, in monocyte transmigration, differentiation, or survival in the vessel wall. However, in high endothelial venules of peripheral lymph nodes, endogenously produced or exogenously applied MCP-1 induces arrest of rolling monocytes (36).

A good example of differential functions of chemokine receptors with overlapping ligand specificity was provided by Weber and colleagues (50). Although RANTES binds to and activates cells through CCR1, 3, and 5, immobilized RANTES leads to arrest of Th1-like T cells, memory T cells (CD45RO⁺), and monocytes on activated endothelial cells through CCR1, but not CCR5 (50). Activation of CCR7 on naïve T cells by SLC generally leads to adhesion via LFA-1 and ICAM-1 (44), but can also trigger $\alpha_4\beta_7$ integrin-dependent arrest on the mucosal addressin adhesion molecule: MAdCAM-1 (35). This suggests that ligation of CCR7 by SLC activates more than one integrin or that different subsets of CCR7⁺ lymphocytes adhere through LFA-1 or $\alpha_4\beta_7$, respectively.

Differential activation was also reported for neutrophils. After activation with CXC chemokines like IL-8, neutrophils adhere through β_2 integrins (27,30). During adjuvant-induced inflammation, mouse neutrophils acquire expression of CCR1 and CCR2, and superfused MCP-1 promoted neutrophil adhesion through an $\alpha_4\beta_1$ -VCAM-1-dependent mechanism (21). It is not clear whether MCP-1 acts as an arrest chemokine, because its surface expression on inflamed endothelium was not demonstrated.

INTRACELLULAR SIGNALING

Integrin-linked kinase (ILK) binds the β_1 integrin cytoplasmic tail and is activated by MCP-1, a CCR2 ligand, with a peak at 15–30 s (13). Interestingly, overexpression of ILK, but not a kinase-dead ILK mutant in THP-1 monocyte-like cells, suppresses $\alpha_4\beta_1$ integrin-dependent adhesion to VCAM-1. There is some evidence that PI3-kinase (PI3K) may be involved in monocyte arrest, because MCP-1 rapidly (within 30 s) activates PI3K α (15). MCP-1-induced monocyte arrest on E-selectin transfected HUVECs was inhibited by the PI3K inhibitors Wort-

mannin and LY294002. Similar effects were also reported for mouse lymphocyte adhesion to low, but not high, site densities of ICAM-1 (7). A constitutively active mutant of PI3K increased adhesion of monocyte-like THP-1 cells (15). Interpretation of these results is complicated by the fact that only soluble-phase but not immobilized MCP-1 has been shown to be an arrest chemokine for monocytes under these conditions (14,20,49). Soluble MCP-1 is unlikely to be present in the blood stream in significant concentrations.

The small G-protein rho has been demonstrated to be involved in arresting lymphoid L1/2 cells (26). L1/2 cells transfected with CXCR1 or the formyl-methionyl-encyl-phenyl-alanine (fMLP) receptor showed nucleotide exchange in rho as early as 10 s after IL-8 or fMLP. Rho activation was required for activation of $\alpha_4\beta_1$ integrin, because *Clostridium botulinum* C3 transferase, which ADP-ribosylates rho, inhibited rapid adhesion of these transfectants to VCAM-1 and of isolated human neutrophils to a fibrinogen-coated surface in response to fMLP, IL-8, and phorbol myristate acetate (PMA). Within the confines of the specificity of this rho inhibitor, these data strongly suggest that rho activation is required for α_4 integrin and possibly also Mac-1-mediated binding of inflammatory cells. While ILK, PI3K, and rho all may play a role in arrest, the signals leading to integrin activation and/or clustering after ligation of chemokine receptors by immobilized chemokines remain to be defined in more detail.

IN VIVO EVIDENCE FOR ARREST CHEMOKINES

The first evidence for an arrest chemokine *in vivo* was provided by Uli von Andrian's lab, who showed that T lymphocytes rolling in high endothelial venules of peripheral lymph nodes arrested by ligation of their chemokine receptor, CCR7, with SLC presented on high endothelial cells (Table 2). Removing SLC removed the arrest function and injecting SLC restored it (44).

More recently, two arrest chemokines have been described which trigger arrest of rolling monocytes. Blocking either RANTES or keratinocyte-derived chemokine (KC) (mouse Gro- α), each results in a 50% reduction of monocyte arrest in freshly isolated and perfused atherosclerotic carotid arteries harvested from apolipoprotein E knockout mice (20,47). In these experiments, expression of RANTES and KC was demonstrated on the endothelial surface; blocking the chemokine or its receptor removed the arrest function; and adding the chemokine back restored monocyte arrest. In lymph node high endothelial venules, MCP-1 can be

presented on the endothelial surface and trigger arrest of rolling monocytes (36). Very recent data suggest arrest chemokines for B cells. SLC, B-lymphocyte chemoattractant (BLC), and SDF-1 may all contribute to the arrest of rolling B cells under physiological conditions (J. Cyster, "Molecular Mechanisms of Leukocyte Trafficking," Keystone Symposium, 2002). These preliminary findings have yet to be confirmed by direct intravital observations.

Topical application of eotaxin promotes $\alpha_4\beta_1$ integrin-dependent eosinophil arrest and transmigration through rat mesenteric venules (34). *In vitro*, eotaxin is a potent arrest chemokine for eosinophils (8). However, it is not known whether eotaxin is an endothelial-expressed arrest chemokine *in vivo*.

CONCLUSIONS

Although a wide variety of chemokines have been suggested to promote adhesion of various types of leukocytes, conclusive evidence is currently only available for naïve lymphocyte arrest via SLC and monocyte arrest via RANTES, Gro- α , and MCP-1. Other arrest chemokines likely exist. The combination of chemokines expressed by the microvascular endothelium at sites of inflammation and by macrovascular endothelium at sites prone to develop atherosclerotic lesions is likely to determine the types of leukocytes recruited into the site. Adhesion molecule-dependent signaling may also contribute to arrest, particularly of neutrophils (29). The specific arrest chemokine requirements for neutrophils, eosinophils, NK cells, dendritic cells, and various types of activated and polarized lymphocytes remain to be determined.

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