Sequential Regulation of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ Integrin Avidity by CC Chemokines in Monocytes: Implications for Transendothelial Chemotaxis

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Abstract. Leukocyte emigration possibly requires dynamic regulation of integrin adhesiveness for endothelial and extracellular matrix ligands. Adhesion assays on purified vascular cell adhesion molecule (VCAM)-1, fibronectin, and fibronectin fragments revealed distinct kinetic patterns for the regulation of very late antigen (VLA)-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) avidity by the CC chemokines monocyte inflammatory protein (MIP)-1$\alpha$, RANTES (regulated on activation, normal T expressed and secreted), or monocyte chemoattractant protein (MCP)-1 in monocytes. CC chemokines induced early activation and subsequent deactivation of VLA-4, whereas upregulation of VLA-5 avidity occurred later and persisted. Controlled detachment assays in shear flow suggested that adhesive strength of VLA-4 for VCAM-1 or the 40-kD fragment of fibronectin (FN40) is more rapidly increased and subsequently reduced by MCP-1 than by MIP-1$\alpha$, and confirmed late and sustained activation of the adhesive strength of VLA-5 for the 120-kD fragment of fibronectin (FN120). Mn$^{2+}$ or the stimulating $\beta_1$ mAb TS2/16 strongly and stably enhanced monocyte binding to VCAM-1 or fibronectin, and locked $\beta_1$ integrins in a high avidity state, which was not further modulated by CC chemokines. Mn$^{2+}$ and mAb TS2/16 inhibited CC chemokine–induced transendothelial migration, particularly chemotaxis across stimulated endothelium that involved VLA-4 and VCAM-1. VLA-4 on Jurkat cells is of constitutively high avidity and interfered with migration across barriers expressing VCAM-1. Low but not high site densities of VCAM-1 or FN40 promoted, while FN120 impaired, $\beta_1$ integrin–dependent monocyte chemotaxis to MCP-1 across filters coated with these substrates. Thus, we show that CC chemokines can differentially and selectively regulate avidity of integrins sharing common $\beta$ subunits. Transient activation and deactivation of VLA-4 may serve to facilitate transendothelial diapedesis, whereas late and prolonged activation of VLA-5 may mediate subsequent interactions with the basement membrane and extracellular matrix.

Transendothelial migration of leukocytes into sites of inflammation is regulated by traffic signal molecules, displayed on activated endothelium, which are considered to act sequentially (7, 59). Tethering and rolling of leukocytes on the vessel wall (39) results in exposure to chemoattractants, such as chemokines presented in the endothelial proximity. Chemokines are divided into two subfamilies according to sequence homology and the arrangement of the first cysteines. CXC chemokines preferentially stimulate neutrophils, and CC chemokines preferentially stimulate monocytes, T lymphocytes, eosinophils, and basophils (2). Chemokines can be found associated with the endothelial surface or complexed to proteoglycan-containing moieties in the subendothelial extracellular matrix (ECM) (52). Immobilized or soluble chemokines induce adhesion of leukocytes to endothelium or purified ligands, implicating their role in integrin activation (14, 29, 59, 60). Integrins bind to Ig superfamily members on endothelium. Like selectins, $\alpha_4$ integrins can support rolling. The integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ mediate rolling on vascular cell adhesion molecule (VCAM)-1 and mucosal addressin cell adhesion molecule (MAdCAM)-1, respectively (1, 5). After activation, $\beta_2$ integrins and $\alpha_4$ integrins on leukocytes support firm adhesion (1, 39). Chemokine gradients can promote diapedesis of leukocytes (29), stimulating diapedesis of leukocytes across filters coated with these substrates.

1. Abbreviations used in this paper: BCECF/AM, 2',7'-bis-2-carboxyethyl-5(6)-carboxyfluorescein-acetoxymethyl ester; ECM, extracellular matrix; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, monocyte inflammatory protein; RANTES, regulated on activation, normal T expressed and secreted; TNF-$\alpha$, tumor necrosis factor $\alpha$; VCAM, vascular cellular adhesion molecule; VLA, very late antigen.
rectional motility that is thought to require reversible integrin–ligand interactions. Diapedesis in vivo requires signaling via G protein–coupled receptors (3, 22, 48, 58). The multistep paradigm provides the combinatorial diversity to explain the selective localization of leukocyte classes and subsets in various inflammatory states (7, 59). However, the mechanisms by which integrins mediate or facilitate leukocyte diapedesis and chemotaxis remain to be elucidated.

Monocytes, eosinophils and lymphocytes but not neutrophils express the β1 integrin very late antigen (VLA)-4 (α4β1, CD49d/CD29) (26), which binds to domains 1 and 4 of VCAM-1, an Ig superfamily member induced by cytokines on endothelium (18, 50, 61, 62). VLA-4 also binds to an alternatively spliced domain of fibronectin, which is distinct from the domain containing the sequence RGD to which the integrin VLA-5 (α5β1) binds (30, 63). In vitro studies suggest that transendothelial migration of monocytes, T lymphocytes, and eosinophils is mainly mediated by interaction of β2 integrins with intercellular adhesion molecule (ICAM)-1 (17, 45, 49, 53). However, interactions of VLA-4 and VCAM-1 contribute to migration of resting T lymphocytes, monocytes, and eosinophils across cytokine-stimulated endothelium, independently of β2 integrins (11, 17, 45, 49). This is also consistent with in vivo studies showing a role of VLA-4 in migration of monocytes and lymphocytes into inflammatory sites or cytokine-induced lesions, after upregulation of endothelial adhesion molecules (31, 32, 66).

Adhesion to ECM glycoproteins such as fibronectin, via VLA-4 and VLA-5, has been implicated in leukocyte migration into inflammatory sites (20). While activating β1 mAb and T cell receptor cross-linking stimulate β1 integrin–mediated adhesion to fibronectin by increasing affinity, phorbol esters affect postreceptor occupancy events, such as cytoskeletal reorganization and spreading (19, 56). Chemokines have also been shown to modulate β1 integrin–dependent binding of lymphocytes to secreted ECM (9, 23).

In contrast with the α5 cytoplasmic domain, the cytoplasmic domain of α4 has recently been described to exhibit distinct properties essential for extravasation, supporting cell migration over adhesion strengthening and spreading (37). Binding of VLA-4 to VCAM-1 on the endothelial surface and binding of VLA-4 and VLA-5 to fibronectin in the basement membrane would be expected to function in distinct steps of monocyte diapedesis. Hence, we speculated that these integrins may differ in the kinetics of activation by chemokines. Here, we show that CC chemokines differentially and selectively regulate adhesiveness of VLA-4 and VLA-5 in monocytes. Transient activation and deactivation of VLA-4 precedes late and persistent activation of VLA-5. This sequential modulation may be crucial in facilitating transendothelial chemotaxis.

Materials and Methods

Reagents and mAbs

The human recombinant chemokines and cytokines macrophage inflammatory protein (MIP)-1α, RANTES (regulated on activation, normal T expressed and secreted), monocyte chemoattractant protein (MCP)-1, interleukin (IL)-8 and tumor necrosis factor α (TNF-α) were from Genzyme Corp. (Cambridge, MA). Accudenz was from Accurate Chemical and Scientific Corp. (Westbury, NY), and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein–acetoxymethyl ester (BCECF/AM) was from Molecular Probes Inc. (Eugene, OR). Other reagents were from Sigma Chemical Co. (St. Louis, MO). The murine IgG myeloma X63, TS1/38 (CD18) (54), and R6.5 (ICAM-1) (57) were purified with protein A. 4B4 (CD29, ascites) (47) was a generous gift from Dr. C. Morimoto (Dana-Farber Cancer Institute, Boston, MA). Purified HAE-2a (VCAM-1) (62) was a generous gift from Dr. T. Tedder (Duke University, Durham, NC). Purified TS2/16 (β1 activating) (27) and A5-PUJ1 (CD49e, ascites) were kind gifts from Dr. M.E. Hemler (Dana-Farber Cancer Institute, Boston, MA). Purified Dreg 56 (L-selectin) was a kind gift from Dr. T. Kishimoto (Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT). Purified HP2/1 (CD49d) (55) was from Amac, Inc. (Westbrook, ME); My4 (CD14) (25) was from Coulter Corp. (Hialeah, FL); and CD32 mAb was from Pharmingen (San Diego, CA).

Blood Cell Isolation

Blood was collected from healthy donors and citrate-anticoagulated. Leukocyte-rich plasma was prepared by 0.6% dextran T500 sedimentation of erythrocytes for 45 min at room temperature. Monocytes were separated from lymphocytes by Accudenz 1.08 hypersonic gradient centrifugation of leukocyte-rich plasma (6, 45). Platelets were removed from monocytes by four washes at 300 g for 7 min. This protocol yielded a population of 80–85% monocytes as analyzed by CD14 staining and light scatter. Monocytes were not activated by this isolation procedure, since expression of L-selectin, which is rapidly shed during monocyte activation, was identical to that on the monocyte fraction in minimally processed whole blood (data not shown), and L-selectin–mediated functions under flow were fully maintained (Alon, R., and T.A. Springer, unpublished data).

Cell Culture

Lymphoblastoid Jurkat T cells transfected with the IL-8 receptor 2 (IL-8R) (41) were maintained in RPMI 1640 medium supplemented with 10% FCS, 50 μg/ml gentamicin, and the selection antibiotic G418 (800 μg/ml; Gibco BRL, Gaithersburg, MD). Viability was >95%, as assessed by trypan blue exclusion.

Cell Adhesion Assay

Cell attachment in stasis to VCAM-1, fibronectin, and BSA, all adsorbed at 2.5 μg/ml, or a 40-kD fragment of fibronectin (FN40), a 120-kD fragment of fibronectin (FN120), and BSA, all adsorbed at 10 μg/ml, was performed as described (44). Soluble recombinant VCAM-1 was a kind gift of Dr. R. Lobb (51). Briefly, proteins were coated onto 96-well microliter plates (Limbro®/Titertek®, ICN Biomedicals, Inc., Aurora, OH). Plates were blocked by addition of 1% human serum albumin (HSA) treated for 2 h at 37°C. Cells were labeled with the fluorescent dye BCECF/AM (1 μg/ml), incubated at the same time with 5% human serum or CD32 (10 μg/ml) for 30 min to block Fe receptors, washed, and resuspended in assay medium. Blocking of Fe receptors improved the inhibition of binding to VCAM-1 by anti-VCAM-1 mAb, by preventing binding of monocytes to mAbs via Fe receptors (data not shown). Some cells were preincubated with blocking mAbs to VLA-4a (HP2/1, 10 μg/ml), VLA-5a (A5-PUJ1, 1:100 ascites), or isotype control (X63, 20 μg/ml) on ice for 20 min, or cytochalasin B (10 μg/ml) for 30 min, and washed. Some cells were preincubated with blocking mAb to VCAM-1 (HAE-2a, 10 μg/ml) and washed. Labeled cells (5 × 10^5 in 50 μl) were added to each well of a ligand-coated microparticle plate with or without chemokines, PMA (100 nM), Mn2+, or TS2/16 and allowed to settle for 10 min on ice. In some experiments, Mn2+ or TS2/16 were added 30 min after stimulation with MIP-1α. Plates were rapidly warmed to 37°C and incubated for indicated periods at 37°C. Fluorescence of input cells was quantified using a fluorescence concentration analyzer (FCA; Idexx, Westbrook, ME). Nonadherent cells were removed by a standardized washing procedure (four cycles, washing volume 200 μl, volume remaining after aspiration 50 μl, with a pressure of 17,000 Pa for Jurkat cells and 21,000 Pa for monocytes) in an automated plate washer (Microplate Autowasher EL-404; Bio-Tek Instruments, Inc., Winooski, VT) using HHM (HBSS, 10 mM Hepes, 1 mM MgCl2, 1 mM CaCl2, pH 7.4). The washing program minimized background binding, as assessed with BSA-coated control wells. Fluorescence of adherent cells.
was analyzed using the FCA. After subtraction of the background binding, which was typically <5% of total, specific binding was calculated as a percentage of input. Data were statistically analyzed using a paired two-tailed t test with Bonferroni corrections, where indicated.

**Controlled Detachment Assay**

1. VCAM-1 was adsorbed at 5 or 10 μg/ml and FN40 or FN120 at 30 μg/ml on plastic dishes, and nonspecific binding was blocked with 2% heat-treated HSA. The plastic dish was assembled as the lower wall in a parallel wall flow chamber and mounted on the stage of an inverted phase-contrast microscope as described (1). Monocytes were washed and resuspended at 5 × 10^6/ml in HMMC medium supplemented with 0.2% HSA, prestimulated with chemokines, PMA (100 nM), TS2/16 (10 μg/ml), or Mn^2+ (1 mM) at 37°C for the indicated periods, and perfused through the chamber at flow rates to obtain defined wall shear stresses. Some cell aliquots were preincubated with VLA-4 mAb (HP2/1, 10 μg/ml), VLA-5 mAb (AS-PUJ1, 1:100 ascites), or isotype control mAb (X63, 10 μg/ml). Experiments were quantitated as described (37). Detachment assays were performed on cells that were allowed to attach in stasis, at low (0.5 dyn/cm^2) or higher shear (1 dyn/cm^2). For detachment, the shear flow was increased in increments every 10 s, and the number of cells per field remaining bound at the end of each 10-s interval was counted to determine the shear resistance of adherent cells. Data were expressed as percentage of cells remaining attached. Data were statistically analyzed using a paired two-tailed t test with Bonferroni corrections, where indicated.

**Flow Cytometry**

Monocytes were stimulated with or without chemokines at 37°C for 15 min in HHMMC with 1% HSA, washed and reacted for 30 min with mAb on ice, washed, stained with a 1:20 dilution of goat anti-mouse IgG FITC (Zymed Labs, Inc., San Francisco, CA), and subjected to scatter-gated flow cytometry in a FACSscan® (Becton Dickinson & Co., Mountain View, CA). Staining of human umbilical cord endothelial cells (HUVEC) with mAbs to ICAM-1 (R6.5, 20 μg/ml), VCAM-1 (HAE-2a, 10 μg/ml), or isotype control (X63, 20 μg/ml) for 20 min on ice. Data were statistically analyzed using a paired two-tailed t test with Bonferroni corrections, where indicated.

## Results

### Effect of CC Chemokines on Adhesion of Monocytes to VCAM-1

To assess the regulation of β1 integrin avidity by CC chemokines, we studied adhesion of isolated human blood monocytes to purified ligands. MIP-1α or RANTES increased specific monocyte binding to VCAM-1 up to threefold at 12 or 15 min (Fig. 1, A and B). Binding was inhibited by mAbs to VLA-4 and VCAM-1 (Fig. 1 B). This indicates that binding was mediated by interaction of VLA-4 with VCAM-1 and that chemokines increase VLA-4 avidity. MIP-1α was most active at 10 ng/ml, and RANTES was most active at 100 ng/ml (Fig. 1 C). Cells were incubated on the adhesive substrate in the continued presence or absence of chemokines for varying times (Fig. 1 A). While binding of unstimulated cells to VCAM-1 slightly increased at 30-60 min, binding to VCAM-1 of monocytes stimulated by MIP-1α or RANTES markedly declined at 30 min and remained lower than binding of unstimulated cells up to 60 min (Fig. 1 A). This contrasted with prolonged activation of VLA-4-mediated monocyte binding to VCAM-1 by MIP-1α (Fig. 1 A) and reflected deactivation of VLA-4 and cell detachment, since binding to VCAM-1 was inhibited >90% by a VLA-4 mAb at all time points (data not shown). The dose–responses were similar for activation and deactivation of binding to VCAM-1 (Fig. 1, C and D). After stimulation with MCP-1, VLA-4-dependent binding of monocytes to VCAM-1 was already reduced at 15 min, further decreased at 30 min, and remained at 50% of resting binding up to 60 min (Fig. 1, A and B). The optimal inhibitory concentration for MCP-1 was 1 ng/ml, as higher and lower concentrations were less active (Fig. 1, C and D; data not shown).

Mn^2+ or the activating β1 mAb TS2/16, which lock β1 integrins in a high avidity state (15, 44), induced an increase in monocyte adhesion to VCAM-1 that was dose-dependent, mediated by VLA-4, and prolonged (data not shown). Adding Mn^2+ or TS2/16 after stimulation with MIP-1α for 30 min rescued binding, indicating that VCAM-1 was still functional when adhesion was reduced by chemokines (Fig. 1 E). Mixing MIP-1α with Mn^2+ or TS2/16 showed that prolonged stimulation by the latter agents was dominant and prevented the deactivation of VLA-4 seen with chemokines at 30 min (Fig. 1 F).

### Effect of CC Chemokines on Adhesion of Monocytes to Fibronectin and Fibronectin Fragments

The kinetics of stimulation by chemokines of binding to fibronectin and fibronectin fragments was similarly examined. MIP-1α or RANTES but not MCP-1 induced a transient increase in adhesion of monocytes to fibronectin by up to 50% at 15 min, but it did not alter adhesion at 30 min, whereas binding was reduced by MCP-1 at 15 or 30 min (Fig. 2 A). At 45 or 60 min and up to 90 min, all chemokines increased binding to fibronectin by up to 50%
Transient effects of CC chemokines on adhesion of monocytes to VCAM-1. (A and B) Kinetics (A) and inhibition by mAb (B) of monocyte binding to VCAM-1. (C and D) Chemokine dose-response for monocyte binding to VCAM-1 at 15 min (C) and 45 min (D). (E) Kinetics of monocyte binding to VCAM-1 in response to PMA and effect of Mn⁺² and activating β1 mAb TS2/16 after stimulation with MIP-1α. (F) Effect of Mn⁺² and activating β1 mAb TS2/16 on regulation of monocyte binding to VCAM-1 by MIP-1α. Monocytes were subjected to adhesion assays on VCAM-1 (adsorbed at 2.5 μg/ml) in the presence of assay medium (control), MIP-1α (10 ng/ml), RANTES (100 ng/ml), MCP-1 (1 ng/ml), 100 nM PMA (A, B, E, and F), or indicated concentrations of chemokines (C and D) at 37°C for indicated periods (A, E, and F), 15 min (B and C), or 45 min (D). Mn⁺² (1 mM) or β1 mAb TS2/16 (10 μg/ml) were added after 30 min of stimulation with MIP-1α (E) or mixed with MIP-1α (F). Some cells were pretreated with isotype control mAb (X63, 10 μg/ml) or VLA-4 mAb (HP2/1, 10 μg/ml), and some wells with VCAM-1 mAb (HAE-2α, 10 μg/ml) (B). Data are mean ± SD of three independent experiments performed in duplicate.

Inhibition with mAbs to VLA-4 or VLA-5 revealed that binding of unstimulated monocytes was mainly mediated by VLA-5 with a <20% contribution by VLA-4 (Fig. 2 B; compare Fig. 2 C with 2 A). Marked inhibition was also achieved by RGD peptide (data not shown). After blocking of VLA-5, binding of monocytes to fibronectin was transiently increased by MIP-1α or RANTES (P < 0.01 vs control), but not by MCP-1 at 15 min, and subsequently decreased with all chemokines (P < 0.05 vs control at 45 min), remaining below binding of control cells up to 60 min (Fig. 2 B). After blocking of VLA-4, binding to fibronectin was unaffected up to 30 min but enhanced with all chemokines at 45 to 60 min (Fig. 2 C). Thus, transient increases in adhesion to fibronectin were VLA-4 dependent, while late increases were mediated by activation of VLA-5. The activation of VLA-5 contrasted with concomitant deactivation of VLA-4. The dose-responses for CC chemokines were identical for the regulation of β1 integrin avidity to fibronectin and VCAM-1 (data not shown). PMA, Mn⁺², or TS2/16 induced a dose-dependent increase in binding to fibronectin that was largely VLA-5 dependent and was prolonged (Fig. 2 D; data not shown). Mixing MIP-1α with Mn⁺² or TS2/16 showed that the immediate and irreversible stimulation by the latter agents was dominant over that induced by CC chemokines (Fig. 2 D).

Consistently, the CC chemokines MIP-1α and RANTES transiently increased monocyte adhesion to a 40-kD fragment of fibronectin (FN40) containing the CS-1 binding site for VLA-4 up to fourfold (Fig. 2 E; P < 0.01 and P < 0.05 vs control for MIP-1α and RANTES, respectively). All chemokines examined reduced adhesion to FN40 at later time points (Fig. 2 E; P < 0.05 vs control at 60 min).

By contrast, under identical conditions, a late and sustained increase in adhesion was induced to a 120-kD fragment of fibronectin (FN120) containing the RGD site for binding of VLA-5 (Fig. 2 F). Binding of unstimulated and stimulated monocytes to FN40 and FN120 was inhibited by VLA-4 and VLA-5 mAb, respectively (data not shown).

The surface expression of VLA-4 and VLA-5 was not affected by CC chemokines, indicating that altered avidity was independent of surface expression (data not shown). Cytochalasin B abrogated chemokine-stimulated increases in adhesion to VCAM-1 and fibronectin (data not shown), implicating actin cytoskeleton–associated events in regulation of avidity. The results show that β1 integrin avidity can be sequentially and differentially modulated by CC chemokines in monocytes. Transient activation was followed by rapid deactivation of VLA-4, while VLA-5 activation occurred later and persisted.

Kinetics of Adhesive Strength of Chemokine-stimulated Monocytes to VCAM-1 and Fibronectin Fragments

To detect rapid changes in adhesive strength to VCAM-1, monocytes were preincubated with chemokines for varying times in suspension, in contrast with static assays above where incubation with the chemokine and substrate were coculture. Monocytes were allowed to attach to VCAM-1 adsorbed at 10 μg/ml in a parallel wall flow chamber at a low wall shear stress (0.5 dyn/cm²) for 1 min, and subsequently subjected to detachment by incremental increases in wall shear stress. This enabled us to test effects of chemokines on adhesion strengthening at early time points. Attachment (~29 cells per field) was prevented by mAbs to VLA-4 but not altered by chemokines (data not shown), indicating that it was VLA-4 dependent and that the efficacy of initial ligand binding by VLA-4 was not modulated by chemokines. A subset of unstimulated cells bound to VCAM-1 with weak adhesive strength, as evident by rapid detachment at low shear stresses (~5 dyn/cm²), whereas another fraction remained firmly attached at high shear stresses (~40 dyn/cm²), indicative of high avidity interactions of VLA-4 with VCAM-1 (Fig. 3 A).
Fewer cells initially attached to VCAM-1 adsorbed at a lower density of 5 µg/ml (~12 cells per field), more cells readily detached at low shear, and few remained bound at high shear (Fig. 3 B).

Examination of early time points with the shear flow assay showed that MCP-1 (1 ng/ml) induced a rapid increase in adhesion strengthening, as shown by shear resistance with a peak at 10 min, but subsequently reduced shear resistance below the level of control cells (Fig. 3, A and C). This demonstrated early activation of VLA-4 avidity and deactivation at time points of 12 min and later, consistent with deactivation at 15 or 20 min in the static assay. Results with MIP-1α in shear flow were also consistent with static assays. MIP-1α (10 ng/ml) transiently strengthened adhesion of monocytes with a peak at 15 min and reduced it below control levels at 20–30 min (Fig. 3 D). Adding Mn2+ or TS2/16 after stimulation with chemokines for 20 min induced shear resistance of >85% of cells, showing that VCAM-1 was functional when adhesive strength was reduced by chemokines (Fig. 3, C and D; data not shown). The chemokine-induced increases in adhesion strength (up to twofold at 8.5 dyn/cm²) were most evident at low or medium shear forces (Fig. 3, C and D; data not shown), suggesting that intermediate avidity states are most sensitive to regulation by chemokines. By contrast, PMA elicited prolonged resistance to detachment of >90% of cells without deactivation at later time points (data not shown). The effect of CC chemokines on VLA-4-dependent adhesion strengthening to VCAM-1 was more marked on lower site density substrates than at 10 µg/ml, since MCP-1 enhanced shear resistance threefold at 8.5 dyn/cm² and sevenfold at 36 dyn/cm² on VCAM-1 at 5 µg/ml (Fig. 3 B).

Cell attachment to VCAM-1 at higher shear (1 dyn/cm²) was twofold less efficient than at 0.5 dyn/cm². CC chemokines failed to modulate adhesion strengthening of this subset (data not shown), suggesting that a subset of monocytes expressing VLA-4 with high affinity for VCAM-1 was selected, and that the adhesive strength of VLA-4 on this subset of cells could not be further regulated by CC chemokines.

Similarly, we studied the kinetics of adhesion strengthening to the fibronectin fragments FN40 and FN120 both adsorbed at 30 µg/ml. Monocytes were allowed to attach in stasis for 2 min and were subjected to detachment assays. Attachment to FN40 (~11 cells per field) and FN120 (~17 cells per field) was inhibited by VLA-4 and VLA-5 mAb, respectively (data not shown). The majority of unstimulated cells attached to FN40 readily detached at low shear stresses, and few remained bound at high shear stresses (Fig. 4 A). Consistent with findings on VCAM-1, shear resistance was rapidly and transiently increased by MCP-1 (1 ng/ml) at 5 and 10 min (up to twofold at 8.5 dyn/cm², P < 0.01 vs unstimulated control), subsequently reduced at 20 min (by up to 50% at 36 dyn/cm², P < 0.05 vs unstimulated control), and was not significantly further modulated at later time points (Fig. 4, A and B). Activation and deactivation of adhesive strength by MIP-1α occurred slightly later than with MCP-1 (data not shown). On FN120, a subset of unstimulated cells detached at low shear stresses, whereas another fraction remained firmly attached at high shear stresses (Fig. 4 C). In accordance with the kinetics of VLA-5 regulation found in static assays, MCP-1 (1 ng/ml) or MIP-1α (10 ng/ml) did not affect shear resistance after pretreatment for up to 30 min, but markedly increased adhesive strength at 40, 50, or 60 min (up to threefold at 8.5 dyn/cm², P < 0.01 vs unstimulated control), indicating that activation of VLA-5 avidity occurred late and was sustained (Fig. 4, C and D; data not shown). By contrast, PMA or Mn2+ induced immediate and prolonged resistance to detachment of >90% of cells (data not shown). This confirmed sequential regulation of VLA-4 and VLA-5 by CC chemokines.
Rapid regulation by CC chemokines of monocyte adhesion strengthening on VCAM-1 under shear flow in controlled detachment assays. (A and B) Detachment profiles on VCAM-1 adsorbed at 10 μg/ml (A) and 5 μg/ml (B) for monocytes prestimulated with MCP-1 (1 ng/ml). (C and D) Kinetics of regulation of adhesiveness on VCAM-1 (10 μg/ml) with MCP-1 (C) and MIP-1α (D). Monocytes were prestimulated with MCP-1 (1 ng/ml, A–C) or MIP-1α (10 ng/ml) (D) at 37°C for the indicated periods or held unstimulated (control) and perfused at 5 × 10^5 cells per ml for 1 min through a flow chamber at 0.5 dyn/cm^2 to allow attachment. Shear was then increased every 10 s in increments, and numbers of cells per field remaining bound at the end of each 10-s interval were determined. In some experiments, Mn^2+ (1 mM) was added 20 min after stimulation with MCP-1 (C) or MIP-1α (D), before shear resistance was determined (dashed lines). Data are mean ± SD of three independent experiments performed in duplicate.

**Role of β1 Integrin Avidity in Transendothelial Chemotaxis**

MIP-1α, RANTES, and, most effectively, MCP-1 induced chemotaxis of monocytes across resting HUVEC via pertussis toxin-sensitive G protein–coupled receptors (Fig. 5 A; data not shown). Treatment of HUVEC with TNF-α for 24 h to upregulate expression of ICAM-1 and VCAM-1 reduced chemotaxis to MCP-1 (Fig. 5 A). This reduction is specific for monocytes and MCP-1 and has been attributed to an impaired chemotactic gradient and/or desensitization due to production of MCP-1 by cytokine-activated HUVEC (12). Inhibition with mAbs to the β2 subunit or ICAM-1 demonstrated a role of CD11/CD18 and ICAM-1 in transendothelial migration of monocytes to MCP-1, MIP-1α, or RANTES (Fig. 5 A; data not shown). Inhibition was also achieved by mAbs to α4 or β1, revealing the involvement of β1 integrins in transendothelial chemotaxis (Fig. 5 A). Little inhibition was seen with α5 mAb. While mAbs to β2 or ICAM-1 were less effective, the mAb to α4 was more effective in inhibiting monocyte migration to MCP-1 across TNF-α–treated than resting HUVEC. VCAM-1 mAb inhibited transmigration to MCP-1 only across TNF-α–treated HUVEC. Inhibition by mAbs to VLA-4 was more marked than by the VCAM-1 mAb with stimulated HUVEC (Fig. 5 A). These data suggest an involvement of VCAM-1 in chemotaxis across stimulated endothelium and interactions of VLA-4 with additional ligands, such as fibronectin.

We studied the role of β1 integrin avidity in transendothelial monocyte chemotaxis. TS2/16 or Mn^2+ dose-dependently inhibited transendothelial chemotaxis of monocytes to CC chemokines both across resting and TNF-α–stimulated HUVEC (Fig. 5 B; data not shown). Transmigration across TNF-α–stimulated HUVEC to MCP-1 was most sensitive to inhibition (Fig. 5 B; data not shown), consistent with the finding that MCP-1 most rapidly and effectively regulated VLA-4 avidity for VCAM-1 (Figs. 1, C and D and 3, C and D). The dose–response for inhibition of chemotaxis by TS2/16 and Mn^2+ correlated with the dose–response for stimulation of β1 integrin affinity (data not shown).

Jurkat T cells were used as a model for cells that constitutively express high avidity VLA-4. Binding to VCAM-1 of IL-8 R2-transfected Jurkat T cells (41) was constitu-
Monocyte Transmigration
Effect of 

with Mn^2+ or activating 131 mAb TS2/16. Monocytes were prein-
compared to uncoated filters (Fig. 7 A). These effects were
up to 60% better monocyte transmigration to MCP-1 as
ligands VCAM-I (2.5 ~g/ml) or FN40 (10 ~g/ml) allowed
their ligands. Interestingly, low site densities of the VLA-4
monocyte chemotaxis across Transwell-filters coated with
We further studied the role of VLA-4 and VLA-5 in
accompanying TNF-a-induced expression of VCAM-1 (2.5 ~g/ml)
in the presence of assay medium (control) or IL-8 (100 ng/ml) at 37°C for indi-
periods, after preincubation with mAbs to VLA-4 (HP2/1,
10 μg/ml) or isotype control (X63, 20 μg/ml). (B) Chemotaxis of
IL-8R2-transfectants to IL-8 (100 ng/ml). Jurkat IL-8R2-transfectants were
preincubated with mAbs to β2, VLA-4a, or isotype control,
and endothelial monolayers were preincubated with
mAbs to ICAM-1 or VCAM-1. Endothelial monolayers were un-
treated (open bars) or treated with TNF-α (100 U/ml) for 24 h
(closed bars). Data are mean ± SD of three independent experiments performed in duplicate.

Discussion
We have found that CC chemokines differentially regulate the avidity of the β1 integrins VLA-4 and VLA-5 in mono-
cytes. CC chemokines transiently increased and subse-
sequently reduced VLA-4-mediated binding of monocytes
to VCAM-1, fibronectin, or FN40, a fibronectin fragment
containing the CS-1 binding site for VLA-4. This con-
trasted with prolonged activation of VLA-4 by phorbol ester and reflected rapid activation and deactivation of
VLA-4, resulting in attachment and subsequent detach-
ment, the VLA-5 ligand FN120 impaired transmigration to
MCP-1 up to 70% at 30 μg/ml (Fig. 7 A). The increase in
transmigration with low density VCAM-1 or FN40 and the
inhibition with FN120 was reversed by preincubation of
monocytes with β1 mAb (Fig. 7 B). An increase in trans-
migration seen with fibronectin adsorbed at a low density
was inhibited to a much greater extent by mAb to α4 than
to α5 (data not shown). Substrates or mAbs hardly af-
fected spontaneous transmigration (Fig. 7, A and B). This
provides further evidence that VLA-4 and its transient
avidity regulation by CC chemokines is specialized in facil-
itating monocyte chemotaxis to MCP-1.

Figure 5. Transendothelial chemotaxis of monocytes. (A) Inhibi-
tion of chemotaxis to MCP-1 (100 ng/ml) with mAbs to adhesion
molecules. (B) Inhibition of chemotaxis to MCP-1 (100 ng/ml)
with Mn^2+ or activating 131 mAb TS2/16. Monocytes were prein-
cubated with or without (control) Mn^2+ or β1 mAb TS2/16 at in-
dicated concentrations for 20 min (B). Endothelial monolayers
on filters were untreated (open bars) or pretreated with TNF-α
(100 U/ml) for 24 h (closed bars). Spontaneous migration in the
absence of MCP-1 was 1.9 ± 0.5% and 2.7 ± 0.3% with resting
and activated HUVEC, respectively. Data are mean ± SD of three independent experiments performed in duplicate.

Effect of β1 Integrin Ligands on MCP-1 Induced
Monocyte Transmigration

We further studied the role of VLA-4 and VLA-5 in
monocyte chemotaxis across Transwell-filters coated with
their ligands. Interestingly, low site densities of the VLA-4
ligands VCAM-1 (2.5 μg/ml) or FN40 (10 μg/ml) allowed
up to 60% better monocyte transmigration to MCP-1 as
compared to uncoated filters (Fig. 7 A). These effects were
not seen at higher substrate densities (Fig. 7 A) and were
most evident with MCP-1 at 1 ng/ml, a concentration opti-
mally modulating VLA-4 avidity (data not shown). In con-
ment of monocytes on substrates bearing ligands for VLA-4. VLA-5 was confirmed to be the predominant receptor for fibronectin in monocytes. In contrast to deactivation of VLA-4, we found a persistent activation of VLA-5 avidity for fibronectin and the RGD-containing fibronectin fragment FN120. We have recently found that CC chemokines and other chemoattractants can also differentially regulate the avidity of β1 and β2 integrins (65). These studies were on eosinophils, which express VLA-4 but little VLA-5. Chemoattractants stimulated transient activation of VLA-4 adhesiveness for VCAM-1 and fibronectin but prolonged activation of Mac-1 (65).

To our knowledge, this is the first demonstration that two integrins sharing the same β subunit are selectively and differentially regulated by the same agonist. This suggests that the integrin α subunits confer differential activation of adhesiveness. Studies in transfectants have provided evidence that the cytoplasmic tail of α4 augments migration but reduces localization into focal adhesion complexes, whereas the α5 cytoplasmic tail appears specialized to promote spreading (10, 37). Since the cytoplasmic domains of α4 and α5 appear to be crucial for their function in locomotion and are also required for stimulation of adhesion by other cellular agonists, such as phorbol ester (36), the differential regulation of VLA-4 and VLA-5 by CC chemokines may be due to differences in their α subunit cytoplasmic domains, e.g., differential interaction with putative regulatory proteins (15).

We have further shown that the avidity of VLA-4 and VLA-5 can be regulated with sequential kinetics by cellular agonists. Early and transient activation of VLA-4 preceded the late and persistent activation of VLA-5 that occurred concomitant with deactivation of VLA-4. The regulation of VLA-4 avidity for VCAM-1, fibronectin, or FN40 by CC chemokines showed similar kinetics, indicating that it was ligand independent. Adhesion of formylmethionyl-leucyl-phenylalanine receptor transfectants to VCAM-1 can be rapidly and transiently upregulated by formylmethionyl-leucyl-phenylalanine (28). In T lymphocytes, CC chemokines induce prolonged β1 integrin-mediated binding to secreted ECM (23) and stimulate avidity of VLA-4 and VLA-5 for fibronectin (9); however, differential contributions of these integrins at different time points were not investigated. Hence, sequential activation of VLA-4 and VLA-5 avidity has not been previously noted.

Overall, the effects of different CC chemokines on VLA-4 and VLA-5 were similar. Furthermore, we have also seen transient activation of VLA-4 in eosinophils (65). The mode of avidity regulation, early and transient or late and prolonged, therefore appears specific for the integrin but not the chemokine or cell type; however, there were some significant differences between chemokines. Controlled detachment assays on VCAM-1 or FN40 in shear flow revealed that MCP-1 induced early modulation of VLA-4 avidity, which was too rapid to be evident in static adhesion assays. Of the chemokines, MCP-1 most rapidly increased adhesive strength of VLA-4 for VCAM-1, and deactivation also occurred most rapidly. Moreover, MCP-1 was most active at low concentrations. This may be attributed to differences in chemokine receptor density, coupling to different G-protein heterotrimers or different kinetics of receptor occupancy (22).

What are the mechanisms for the regulation of VLA-4 and VLA-5 avidity by CC chemokines? We found that monocyte attachment to VCAM-1 in shear flow was not altered by chemokines, indicating that the initial binding of VLA-4 to VCAM-1 was not changed. This implies that regulation of VLA-4 avidity by CC chemokines occurred after ligand binding. Inhibition of chemokine-stimulated monocyte adhesion to VCAM-1 and fibronectin by cytochalasin B suggested that assembly or reorganization of the actin cytoskeleton was involved in activation of both VLA-4 and VLA-5 avidity. Consistent with our findings, MIP-1β has been shown to induce lymphocyte adhesion to VCAM-1 substrates without increasing affinity of VLA-4 for soluble VCAM-1 (35). Postreceptor occupancy events have also been implicated in stimulation by phorbol ester of adhesion to fibronectin that is mediated by VLA-4 and VLA-5 but not accompanied by changes in ligand binding affinity (13, 19). Recently, “EF-hand”-like divergent cation sites have been defined in the extracellular domain of α4 that are essential for clustering and adhesive strength of VLA-4 but not for monovalent ligand binding (Puigades, C., S-K. Krueff, R. Alon, A. Masumoto, L. Burke, T.A. Springer, L.B. Chen, R.R. Lobb, and M.E. Hemler, manuscript submitted for publication). Hence, VLA-4 adhesiveness may be activated by juxtaposition or lateral multimerization in conjunction with changes in cytoskeletal associations (15).

In monocytes stimulated with MIP-1α on VCAM-1, immunofluorescence showed uropod formation, actin polymerization, and asymmetrical distribution of VLA-4 into uropod-like structures (R. Pujades, C., and T.A. Springer, unpublished data). Thus, regulation of avidity may also be associated with spatial redistribution of VLA-4.

We assessed the contribution of integrins to transcendothelial chemotaxis of monocytes. Our data show that mAb to β1, β2, α4, or α5 independently inhibited transcendothelial chemotaxis. The relative importance of β2 integrins and ICAM-1 was reduced, that of VLA-4 was increased, and that of VCAM-1 was only evident across activated HUVEC. Activation of endothelium has recently been shown to support VLA-4-mediated monocyte transmigration (11). Inhibition was more marked with mAbs to VLA-4 than to VCAM-1, suggesting that VLA-4 can interact with...
ligands other than VCAM-1, as shown for spontaneous transmigration of monocytes (46). Both VLA-4 and VLA-5 may interact with fibronectin secreted by HUVEC into the underlying ECM (33). Monocytes have been reported to use either β2 integrins or VLA-4 during spontaneous transendothelial migration or chemotaxis (11, 45). However, our data suggest that these integrins may function in concert or at distinct steps to synergistically achieve optimal transendothelial chemotaxis, particularly across activated endothelium. This is consistent with an essential role of VLA-4 in emigration of monocytes to acute inflammatory sites in vivo (32, 66).

We studied how the avidity state of β1 integrins affects the capacity for regulation by CC chemokines and transendothelial chemotaxis. Locking β1 integrins in a state of high affinity using activating β1 mAb TS2/16 or Mn2+ (21, 44) induced immediate and prolonged activation of VLA-4 and VLA-5 in monocytes, which was dominant over the modulation of avidity (e.g., VLA-4 deactivation) by CC chemokines. This was associated with strong inhibition of transendothelial chemotaxis, particularly across stimulated HUVEC and to MCP-1. Similarly, chemoattractants transiently activate VLA-4 in eosinophils (Weber, C., J. Kitayama, and T.A. Springer, manuscript submitted for publication), and chemokine-induced migration of eosinophils across HUVEC- or fibronectin-coated filters is blocked by the stimulating mAb 8A2, which appears to freeze β1 integrins in a high avidity state (38). Therefore, the modulation of VLA-4 avidity also appears to be required for eosinophil transendothelial chemotaxis. Expression of VLA-4 in different cellular environments results in multiple activation states that differ in constitutive avidity and capacity for stimulation of binding to different ligands (9, 35, 36, 44). As a model for cells expressing highly active VLA-4, we used Jurkat IL-8R2-transfectants (41). The CXC chemokine IL-8 only moderately increased the constitutively high adhesion of these cells to VCAM-1, without deactivation of VLA-4 at later time points. Migration of Jurkat IL-8R2-transfectants to IL-8 across stimulated HUVEC was impeded by binding of the constitutively avid VLA-4 to VCAM-1, as shown by enhancement of migration by mAb to both VCAM-1 and VLA-4. Monocytes treated with TS2/16 and subjected to transendothelial chemotaxis were arrested on the apical surface of HUVEC monolayers without protruding into filter pores (Weber, C., and T.A. Springer, unpublished data). Our data suggest that high avidity states of VLA-4 interfere with migration, especially across barriers expressing VCAM-1.

Low or intermediate avidity of VLA-4 may be required to allow dynamic regulation by CC chemokines during transendothelial chemotaxis of leukocytes. In monocytes, the avidity of VLA-4 for fibronectin or FN40 appeared to be more substantially activated than for VCAM-1. This difference has been suggested to be characteristic of cells expressing VLA-4 with relatively low constitutive activity (44). Controlled detachment assays on VCAM-1 enabled us to differentiate between two major subsets of monocytes, the first detaching readily at low or medium shear, the other remaining bound at higher shear. Adhesion strengthening was optimally upregulated in the subset with low or medium adhesive strength. The shear resistance of cells selected by attachment at higher shear was not increased or decreased by CC chemokines. This confirms that high constitutive avidity impairs, whereas low constitutive avidity favors regulation of VLA-4. It has been predicted that intermediate adhesion strength leads to maximal motility (16). The properties of VLA-4 described herein are consistent with a role in highly motile rather than stationary leukocyte phenotypes (40).

The transient nature of VLA-4 avidity regulation and the inhibitory effects of high avidity VLA-4 states on transendothelial migration suggested that VLA-4 is specialized in monocyte motility. Indeed, interaction of VLA-4 with its ligands VCAM-1, FN40, or fibronectin allowed better monocyte chemotaxis to MCP-1 across filters coated with these substrates at low but not high site densities, without affecting spontaneous transmigration. As we have found that adhesion strengthening on VCAM-1 was more markedly regulated on lower site density substrates, this infers that the regulation of VLA-4 avidity for its ligands by CC chemokines may serve to facilitate transendothelial chemotaxis. In contrast, interaction of VLA-5 with FN120 impaired transmigration. However, reduction of monocyte chemotaxis across HUVEC or low density fibronectin-coated filters with α5 mAb suggested an involvement of VLA-5. Taken together, this may indicate that late activation of VLA-5 may help complete transmigration if preceded by or in concert with interactions of VLA-4 with its ligands.

The regulation of β1 integrin avidity by CC chemokines may have important functional implications for leukocyte emigration and must be choreographed with regulation of β2 integrin avidity in transendothelial chemotaxis. VLA-4 and β2 integrins are differentially used during adhesion and transendothelial migration of T lymphocytes (49), and distinct functions have been found for these integrins in the interaction of monocytes and T lymphocytes with endothelial monolayers in shear flow (42, 43). VLA-4 participates in adhesion in shear flow to stimulated endothelium, whereas β2 integrins mediate firm adhesion and transmigration. A recently proposed version of the multi-step model defined a role for α4 integrins in bridging between selectins and β2 integrin-mediated events (4). Both α4β1 and VLA-4 β4 support rolling and attachment in flow on ManCAM-1 and VCAM-1, respectively (1, 5). Transient activation and deactivation of VLA-4 avidity by chemokines may follow initial tethering and rolling of leukocytes through selectins and α4 integrins, and may be important either in migration laterally along endothelium to an interendothelial cell junction where diapedesis can occur, or in diapedesis itself. Interaction of β2 integrins with ICAM-1 and other ligands on endothelium may be most important in transendothelial migration. Late and prolonged activation of VLA-5 avidity for fibronectin may support interactions of leukocytes with the underlying basement membrane and with the ECM. This would comply with the sequential regulation of VLA-4 and VLA-5 avidity by chemokines. The specialization among β1 integrins in cellular modulation of their adhesive functions that we have demonstrated may be important for accomplishing the complex process of leukocyte extravasation.

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