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The Affinity of Integrin $\alpha_4\beta_1$ Governs Lymphocyte Migration[1]

David M. Rose,* Valentin Grabovsky,† Ronen Alon,† and Mark H. Ginsberg[2]*

The interaction of integrin $\alpha_4\beta_1$ with endothelial VCAM-1 controls the trafficking of lymphocytes from blood into peripheral tissues. Cells actively regulate the affinity of $\alpha_4\beta_1$ for VCAM-1 (activation). To investigate the biological function of $\alpha_4\beta_1$ activation, we isolated Jurkat T cell lines with defective $\alpha_4\beta_1$ activation. Using these cells, we found that $\alpha_4\beta_1$-stimulated $\alpha_L\beta_2$-dependent cell migration was dramatically reduced in cells with defects in $\alpha_4\beta_1$ activation. These cells required 20 times more VCAM-1 to promote $\alpha_L\beta_2$-dependent cell migration. This defect was at the level of $\alpha_4\beta_1$ affinity as an activating $\alpha_4\beta_1$ Ab rescued $\alpha_4\beta_1$-stimulated $\alpha_L\beta_2$-dependent migration. In contrast, migration of $\alpha_4\beta_1$ activation-defective cells on VCAM-1 alone was enhanced at higher VCAM-1 densities. Thus, $\alpha_4\beta_1$ activation determines a set point or threshold at which VCAM-1 can regulate $\alpha_L\beta_2$-dependent as well as $\alpha_4\beta_1$-dependent cell migration. Changes in this set point may specify preferred anatomical sites of integrin-dependent leukocyte emigration from the bloodstream. *The Journal of Immunology,* **2001, 167: 2824–2830.**

The exit of leukocytes from the vasculature is essential to the development of the immune system, to leukocyte recirculation, and to the control of the inflammatory response (1). Defined subpopulations of leukocytes emigrate at specific sites to produce characteristic leukocyte repertoires in peripheral tissues (2, 3). These emigration decisions are controlled by multiple factors, including leukocyte rolling receptors (e.g., selectins) and the density of their ligands (e.g., P-selectin glycoprotein ligand), site-specific chemokines, and cell migration mediated through the interaction of leukocyte integrins with vascular ligands (4, 5). These three parameters form a leukocyte area code that specifies the composition of leukocyte populations at extravascular sites (4).

Changes in the function of leukocyte integrins may also contribute to the control of leukocyte emigration from blood vessels. Two general mechanisms have been described by which integrin-mediated adhesion is regulated: 1) alterations in integrin affinity for extracellular ligands (activation) and 2) affinity-independent mechanisms such as changes in receptor mobility (6). α_4 integrins play a major role in controlling leukocyte emigration, but the role of activation in α_4 integrin function has been questioned (7). Recent studies established that α_4 integrins undergo active affinity modulation (8). Furthermore, NK cells express constitutively active $\alpha_4\beta_1$, whereas the bulk of resting T cells expresses inactive $\alpha_4\beta_1$. Agonists can activate $\alpha_4\beta_1$ on memory, but not naive T cells. Moreover, integrin affinity can control multiple cellular responses in addition to cell adhesion. Thus, the activation of integrin $\alpha_4\beta_1$ is leukocyte type specific, but its role in the control of leukocyte functions is unclear.

α_4 integrins have potent signaling functions that complement their capacity to mediate cell adhesion. For example, α_4 integrins strongly promote cell migration (9). Indeed, engagement of α_4 integrins by trace quantities of VCAM-1 markedly stimulates β_2 integrin-mediated cell adhesion and migration (10, 11). In this sense, VCAM-1 is an agonist for $\alpha_4\beta_1$. The capacity of agonists to initiate cellular responses is a function of the affinity of their cellular receptors. The $\alpha_4\beta_1$ ligand, VCAM-1, is variably expressed at most vascular sites depending on the presence of inflammatory responses (12, 13). Thus, affinity regulation of this integrin might play a role in determining the threshold or sensitivity of leukocytes to stimulation by VCAM-1.

$\alpha_4\beta_1$ affinity modulation could potentially regulate a number of cellular functions such as adhesion, migration, and signaling. In the present study, we have assessed the biological role of activation of integrin $\alpha_4\beta_1$ by deriving novel cell lines that are incapable of activating $\alpha_4\beta_1$. Using these cell lines, we find that $\alpha_4\beta_1$ affinity determines the set point at which 1) VCAM-1 can stimulate $\alpha_L\beta_2$-dependent cell migration on ICAM-1 and 2) $\alpha_4\beta_1$-dependent migration onVCAM-1 is regulated. In contrast, the activation of $\alpha_4\beta_1$ had little impact on static cell adhesion or adhesion in shear flow. The data suggest that the affinity state of α_4 integrins governs the selection of preferred sites of integrin-dependent leukocyte transmigration.

Materials and Methods

Cells

The Jurkat E6-1 T leukemic cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (BioWhittaker), 1% glutamine, 1% penicillin, and 1% streptomycin (Sigma, St. Louis, MO).

Reagents

The anti-human β_1 mAb 8A2 was a generous gift from N. Kovach and J. Harlan (University of Washington, Seattle, WA). The anti-human α_4, HP2/1; anti-human α_5, SAM1; and anti-human β_1, K20 Abs were purchased from Immunotech (Westbrook, ME). The anti-human β_2 mAb hybridoma cell line TS1/18 was obtained from American Type Culture Collection and was used to generate ascities fluid. The cDNA encoding the CS-1 region of fibronectin fused to GST was a gift from J. W. Smith (Burnham Institute, La Jolla, CA). The expression and purification of this fusion protein have been previously described (14).

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Construction and expression of VCAM-1- and ICAM-1-Ig fusion proteins

The cDNA for human VCAM-1 was a generous gift from T. Collins (Harvard University, Cambridge, MA). The coding sequence of the complete seven Ig domains of the extracellular region of VCAM-1 was PCR amplified and cloned into the *Nhe*I site of plasmid pB4Ig (a gift from R. Cobb, Tanabe Research Laboratories, San Diego, CA), which contains the human Fc coding sequence. The resulting VCAM-Ig fusion construct was excised with *Kpn*I and cloned into pcDNA3.1$^-$ (Invitrogen, Carlsbad, CA). The resulting construct was transfected into Chinese hamster ovary (CHO)[3] cells, and stable cell lines were isolated by selection in G418. A VCAM-Ig-expressing clonal cell line was isolated by limited dilution cloning and screening supernatant for VCAM-Ig production with a VCAM-1 ELISA (R&D Systems, Minneapolis, MN). Recombinant protein was purified from CHO cell supernatant using a protein A column. Similarly, an ICAM-Ig fusion construct, encoding the N-terminal two Ig-like domains of ICAM-1 (a generous gift from D. L. Simmons, CRF Laboratories, University of Oxford, Oxford, U.K.), was subcloned into pcDNA3.1$^-$ and transfected into CHO cells. ICAM-Ig fusion protein was isolated from the supernatants produced by a clonal cell line by protein A affinity chromatography.

Soluble VCAM-Ig-binding assay

Cells (5×10^5) were resuspended in a modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 1 mg/ml glucose, and 1 mg/ml BSA) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The VCAM-Ig fusion protein was added at a final concentration of 100 nM and incubated for 30 min at room temperature. Cells were washed twice in Tyrode's buffer and resuspended in the same buffer containing FITC-conjugated donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA) at a 1/100 dilution. After a 30-min incubation at 4°C, cells were washed twice, and bound Ab was detected using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest software.

Generation and isolation of Jurkat mutants

Jurkat cells were treated with ethyl methane sulfonate (EMS; 200 μg/ml) or ICR-191 (1.5 μg/ml; Sigma) for 24 h. After 5 days in culture, soluble VCAM-1 (sVCAM-1)-binding assays were performed, and low sVCAM-1-binding cells were isolated by cell sorting using a FACStar^{Plus} flow cytometer (BD Biosciences) into 96-well tissue culture-treated plates (Costar, Corning, Corning, NY). Isolated clonal lines were sequentially reassayed for α_4 expression with mAb HP2/1 and sVCAM-1 binding by flow cytometry. Jurkat lines with normal α_4 expression and low sVCAM-1 binding were used for further analysis.

Cell adhesion assays

Ninety-six-well Immulon 2HB plates (Dynex Technologies, Chantilly, VA) were incubated with indicated concentrations of VCAM-1 or the CS-1-containing fragment of fibronectin overnight at 4°C. Afterward, wells were blocked with 2% BSA in PBS for 30 min at room temperature. Cells in a modified Tyrode's buffer were added to wells and allowed to adhere for 40 min at 37°C. Nonadherent cells were washed off with Tyrode's buffer. Adherent cells were stained with 0.5% crystal violet stain in 20% methanol. The cell-incorporated crystal violet was solubilized with 10% acetic acid and measured in a microplate reader (Molecular Devices, Sunnyvale, CA) set at 560 nm.

Cell migration assay

Cell migration was assayed in a modified Boyden chamber assay system. Transwells (Costar; Corning) polycarbonate membranes containing 3-μm pores were incubated with VCAM-1 and/or ICAM-1 in 0.1 M $NaHCO_3$ (pH 8) overnight at 4°C. Membranes were blocked with 2% BSA in PBS for 30 min at room temperature. A total of 2×10^5 cells in RPMI 1640 with 10% FCS was added to the top chamber. Stromal cell-derived factor-1α (R&D Systems) at a final concentration of 15 ng/ml was added to the bottom chamber. Cells were allowed to migrate for 4 h at 37°C. Cells in the bottom chamber were enumerated with a hemocytometer.

VCAM-1 ELISA

The amount of recombinant VCAM-1 bound to the polycarbonate membranes of the transwells was measured by ELISA. Briefly, transwell membranes were coated as described above for cell migration assay, except in the case of the sVCAM-1 studies in which membranes were coated with ICAM-1 and blocked with BSA before addition of sVCAM-1. Afterward, the membranes were washed and anti-human VCAM-1 Ab, P8B1 (1/3000 diluted ascites) was added and incubated for 4 h at room temperature. After extensive washes, an HRP-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA) was incubated with the membranes for 2 h at room temperature. Bound Ab was detected with an 80 mM citrate phosphate buffer (pH 5) containing *o*-phenylenediamine and hydrogen peroxide using a Molecular Devices ELISA plate reader set at 490 nm.

Laminar flow assays

A polystyrene plate coated with sVCAM-1 (affinity-purified seven-domain human VCAM-1, a gift from R. Lobb, Biogen, Cambridge, MA) was assembled in a parallel plate laminar flow chamber (260-μm gap) and mounted on the stage of an inverted phase-contrast microscope (Diaphot 300; Nikon, Tokyo, Japan), as previously described (15). Jurkat cells were perfused at 10^6 cells/ml of binding medium (HBSS containing 2 mg/ml BSA and 10 mM HEPES, pH 7.4, supplemented with Ca^{2+} and Mg^{2+}, each at 1 mM) at the desired shear stress generated with an automated syringe pump (Harvard Apparatus, Natick, MA). Cellular interactions on a field of view of 0.34 mm^2 were visualized with a $\times$10 objective and manually quantified by analysis of images directly from the monitor screen. The motion of each interacting cell was monitored for 10 s following its initial tethering, and three categories of tethers were defined: transient, if cells attached briefly ($<$2 s) to the substrate; rolling, if cells tethered and rolled on the substrate $>$5 s with a velocity $>$1 μm/s; arrest, if following rolling or immediately after tethering, cells came to a full arrest and remained stationary on the substrate for at least 20 s. The number of tethers for each category was divided by the flux of freely flowing cells. For calculations of cell flux, only the fraction of perfused cells that came into close proximity with the substrate, and therefore was potentially capable of interacting with the substrate, was considered.

Controlled flow detachment assays were performed on cells that were settled at stasis on ligand-coated plates for 1 min and then were subjected to wall shear stresses increased stepwise every 5 s (by a programmed set of flow rates delivered by the syringe pump). At the end of each 5-s interval of the increase in shear stress, the number of cells that remained bound was expressed relative to the number of cells originally settled on the substrate in stasis. All assays were performed at room temperature. To study peptide inhibition of $\alpha_4\beta_1$-mediated tethering events, cells were suspended in binding medium with 0.5 mM octapeptide EILDVPST (containing the tripeptide very late Ag-4-binding motif leucine-asparatate-valine, LDV) or its control analogue EIDVLPST for 5 min, and then perfused unwashed through the flow chamber over the VCAM-1-coated substrate.

Results

Isolation of Jurkat cell lines with defective integrin $\alpha_4\beta_1$ activation

To evaluate the functional importance $\alpha_4\beta_1$ affinity modulation, we generated a panel of variant Jurkat T cells with defects in $\alpha_4\beta_1$ activation (Act. Defect. Jurkat). Jurkat cells were chemically mutagenized with either EMS or ICR-191, and α_4-expressing cells were selected for reduced sVCAM-1 binding by flow cytometry. Clonal lines were isolated that expressed α_4, but failed to bind sVCAM-1. The characterization of a representative mutant line, JD6, is shown in Fig. 1. In contrast to wild-type Jurkat cells, the mutant cells showed marked reduction in constitutive sVCAM-1 binding, but binding was reconstituted in the presence of the exogenous β_1 integrin-activating mAb, 8A2. Furthermore, α_4, α_5, β_1, and β_2 integrin subunit expression was similar to that of wild-type Jurkat cells. These results indicate that these mutant lines have a defect in $\alpha_4\beta_1$ activation, which is not due to a change in integrin expression nor a defect in the integrin's ligand binding site. Three independent cell lines were isolated from three different mutagenesis experiments, two with EMS and one with ICR-191. Similar results were obtained with all three mutant lines in the experiments to be described.

The rescue of sVCAM-1 binding in the mutant lines by mAb 8A2 indicates that the intrinsic ligand-binding capacity of the $\alpha_4\beta_1$ is retained and the defect impacts the activation process. Defective

[3] Abbreviations used in this paper: CHO, Chinese hamster ovary; EMS, ethyl methane sulfonate; SDF, stromal cell-derived factor; sVCAM, soluble VCAM.

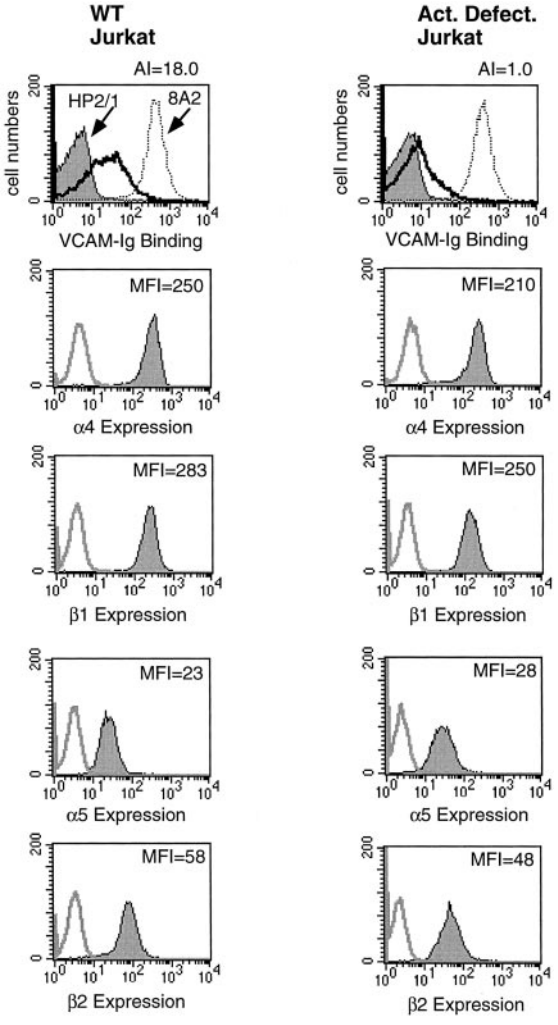

FIGURE 1. Characterization of mutant Jurkat T cell lines. *Top panels*, Histograms of sVCAM-1 binding, as measured by flow cytometry. Solid lines are sVCAM-1 binding in a modified Tyrode's buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Filled histograms are the binding with the addition of anti-α_4 Ab, HP2/1. Dotted lines are binding with the addition of activating β_1 Ab, 8A2. Quantification of sVCAM-1 binding was assessed as an activation index (AI) defined as $100 \times (F_o - F_r)/(F_{max} - F_r)$, in which F_o is mean fluorescence intensity of sVCAM-1 binding, F_r is fluorescence intensity in the presence of mAb HP2/1, and F_{max} is the fluorescence intensity in the presence of mAb 8A2. Subsequent panels are flow cytometric analysis of integrin expression. Binding of specific mAb for α_4 (HP2/1), β_1 (8A2), α_5 (SAM1), and β_2 (TS 1/18) was detected with a FITC-conjugated goat anti-mouse IgG (filled histograms). Open histograms are the binding of purified mouse IgG. Geometric mean fluorescence intensity (MFI) is shown in each panel. Results are representative of three separate experiments with similar results.

activation could be due to mutations in either subunit of integrin $\alpha_4\beta_1$ or a signaling molecule that is required for $\alpha_4\beta_1$ activation. To distinguish between these two possibilities, mutant lines were transfected with wild-type α_4 and β_1 integrin subunits. Failure to rescue sVCAM-1 binding by introduction of wild-type $\alpha_4\beta_1$ indicates that cells have a defect in signaling machinery needed to activate $\alpha_4\beta_1$. The mutant lines were transiently transfected with green fluorescent protein (as a markers of transfection) and cDNAs encoding either α_4, β_1, or a combination of subunits, and sVCAM-1-binding assays performed by flow cytometry. In all three lines examined, transfection of exogenous $\alpha_4\beta_1$ failed to rescue sVCAM-1 binding (data not shown). This result indicates that the defect in integrin activation is not ascribable to integrin mutations, but rather is due to a defect in cellular mechanisms required for activation of $\alpha_4\beta_1$.

The affinity state of $\alpha_4\beta_1$ controls cell migration

$\alpha_4\beta_1$ integrins can regulate adhesion and migration mediated by β_2 integrins (10, 11, 16). Under these conditions, VCAM-1 acts as agonist that regulates β_2 integrin functions. The regulation of integrin function by soluble agonist, such as chemokines, is a function of the affinity of the agonist receptor. By analogy, we reasoned that the capacity of VCAM-1 to stimulate β_2 integrins would be a function of the affinity state of $\alpha_4\beta_1$ (Fig. 2*A*). To examine $\alpha_4\beta_1$-stimulated β_2-dependent migration, Jurkat cells were allowed to migrate on a substrate coated with 200 μg/ml ICAM-1, a ligand for β_2 integrins. We examined the effect of addition of trace quantities of VCAM-1 (0.1–10 μg/ml). Under these conditions, the filters were coated with ~250 molecules/μm^2 of ICAM-1, and the addition of small quantities of VCAM-1 did not significantly displace ICAM-1 (Fig. 2*B*).

Trace quantities of VCAM-1 stimulated Jurkat migration on ICAM-1 (Fig. 2*C*). The stimulated migration was completely blocked with an anti-β_2 Ab, TS1/18, indicating that the migration was β_2 dependent. Furthermore, the stimulated migration was blocked with an anti-VCAM-1 Ab, P8B1 (Fig. 2*C*), as well as an anti-α_4 Ab, HP2/1 (data not shown). As an alternative means of assessing VCAM-1 *trans* activation of β_2-dependent cell migration, we used sVCAM-1 to stimulate migration across modified Boyden chambers coated with ICAM-1 alone. As shown in Fig. 2*D*, the addition of sVCAM-1 to Jurkat cells stimulated β_2-dependent cell migration. ELISA confirmed that negligible amounts of the sVCAM-1 became absorbed to the membranes under these conditions (Fig. 2*D*). These results indicate that the VCAM-1 acted as an agonist, stimulating β_2-dependent migration by binding to $\alpha_4\beta_1$.

To investigate the role of $\alpha_4\beta_1$ affinity modulation in α_4-stimulated β_2-dependent migration, the migration of activation-defective JD6 cells on a mixed substrate of ICAM-1 and VCAM-1 was examined. In contrast to wild-type Jurkat cells, the stimulated migration of activation-defective cells required a more than 20-fold higher concentration of VCAM-1 (Fig. 3*A*). Since these mutant lines were derived by chemical mutagenesis, it is possible that cellular changes other than those affecting $\alpha_4\beta_1$ affinity could account for the decreased sensitivity of β_2-dependent migration to VCAM-1 stimulation. To test this possibility, we used an activating β_1 Ab, 8A2, to reconstitute high-affinity $\alpha_4\beta_1$ on the mutant lines. In the presence of 8A2, VCAM-1-stimulated β_2-dependent migration was identical in the mutant and wild-type Jurkat T cell lines (Fig. 3*B*). This rescue in function was not observed when a nonactivating anti-β_1 Ab, K20, was used (data not shown). Thus, the capacity of differing quantities of VCAM-1 to stimulate β_2 integrin-dependent events is a function of the affinity state of $\alpha_4\beta_1$.

We next questioned whether the mutant lines might have a general migration defect. In the absence of VCAM-1, the migration of wild-type and activation-defective Jurkat cells on ICAM-1 was similar (Fig. 4*B*). The dose-response curve was biphasic, with maximal migration occurring at an ICAM-1-coating concentration of 10 μg/ml. Furthermore, the migration of wild-type Jurkat cells on VCAM-1 in the absence of ICAM-1 was biphasic, with maximal migration occurring at a VCAM-1-coating concentration of ~10 μg/ml (Fig. 3*A*). However, the migration of the mutant lines on VCAM-1 was augmented relative to wild-type cells, and maximal migration occurred at a VCAM-1-coating concentration of ~40 μg/ml. Thus, the mutant cells do not have a general defect in

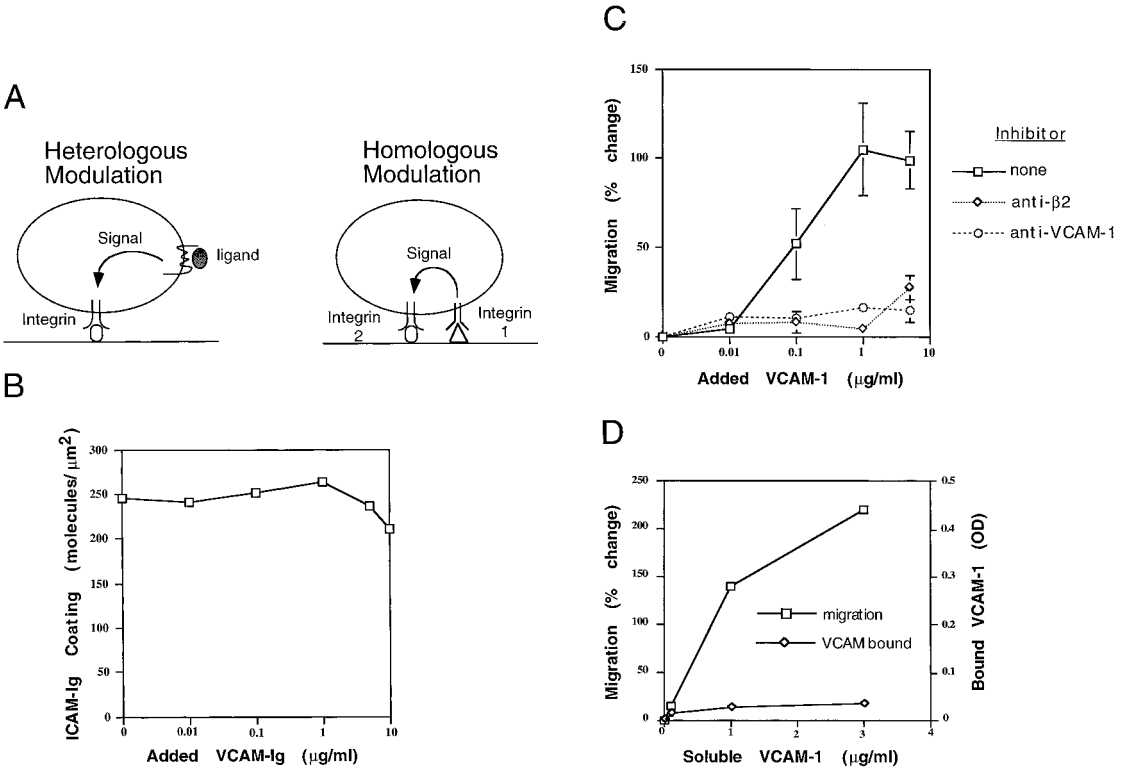

FIGURE 2. $\alpha_4\beta_1$ integrin interaction with VCAM-1 stimulates β_2 integrin-dependent Jurkat T cell migration. *A*, Agonist modulation of integrin function. In heterologous modulation, a cell surface receptor (e.g., chemokine receptor) activates a signaling pathway, which modulates integrin function. In homologous modulation, an integrin (integrin 1), engages ligand and activates a signaling pathway that modulates the function of a second integrin (integrin 2). *B*, Quantification of ICAM-1 coating on polycarbonate membranes of transwell migration chambers. ICAM-1 was radiolabeled with ^{125}I. Membranes (0.33 cm^2) were incubated with ^{125}I-labeled ICAM-1 mixed with the indicated amounts of VCAM-1. Specific activity of ^{125}I-labeled ICAM-1 was used to calculate ICAM-1 coating expressed as molecules per square micrometer. *C*, Migration of wild-type Jurkat T cells. Transwell membranes were coated with 200 μg/ml ICAM-1 and indicated concentrations of VCAM-1. Jurkat cells were added to the top chamber either untreated or with the addition of anti-β_2 Ab (TS 1/18) (20 μg/ml) or anti-VCAM-1 (P8B1) (20 μg/ml). SDF-1α (15 ng/ml) was added to the bottom chamber, and cells were allowed to migrate for 4 h at 37°C. Cells in the bottom chamber were enumerated, and migration was expressed as a percent change relative to no VCAM-1 addition. Results are the mean ± SEM of three separate experiments. *D*, sVCAM-1 stimulates β_2-dependent migration of Jurkat T cells. Transwell membranes were coated with ICAM-1 (200 μg/ml). Jurkat cells were pretreated for 5 min with the indicated concentrations of sVCAM-1 and then added to the top chamber of the transwell. SDF-1α (15 ng/ml) was added to the bottom chamber, and cells were allowed to migrate for 4 h at 37°C. Cells in the bottom chamber were enumerated, and migration was expressed as a percent change relative to no VCAM-1 addition. VCAM-1 bound to the transwell membrane was measured by ELISA, as described in *Materials and Methods*, and expressed as OD units read at 490 nm. For reference, an OD of 0.46 U was observed when the membranes were directly coated with VCAM-1 at a concentration of 1 μg/ml. Results shown are representative of three separate experiments.

integrin-dependent cell migration. Furthermore, the $\alpha_4\beta_1$-dependent migration on different densities of VCAM-1 is a function of $\alpha_4\beta_1$ affinity state. These effects of $\alpha_4\beta_1$ activation on cell migration were similarly observed in the two other mutant Jurkat lines (Table I).

Disruption of $\alpha_4\beta_1$ activation has little effect on cell adhesion under static or flow conditions

We next examined the adhesive properties of the α_4 activation-defective Jurkat lines. Under static conditions, adhesion of these lines to a wide concentration range of coating concentrations of VCAM-1 or the CS-1 fragment of fibronectin was not markedly different from wild-type Jurkat cells (Fig. 5). For both cell lines, the adhesion was α_4 dependent, as it was blocked with anti-α_4 Ab, HP2/1. Thus, $\alpha_4\beta_1$ activation plays little role in regulating static Jurkat cell adhesion.

Integrin $\alpha_4\beta_1$ supports dynamic and reversible tethering and rolling of cells in flowing blood (17). At a shear flow of 1 dyne/cm^2, however, there was no significant difference in the number of activation-defective Jurkat cells rolling and subsequently arresting on VCAM-1 as compared with wild-type Jurkat cells (Fig. 6*A*). Furthermore, when increasing shear force was applied, there was no difference in the detachment of mutant and wild-type cells from VCAM-1 (Fig. 6*B*). Thus, the activation-defective cells exhibit similar adhesion strengthening as wild-type cells. Chen and coworkers (15) previously implicated the importance of high-affinity $\alpha_4\beta_1$ in adhesion strengthening of cells under flow conditions. This was partially based on the ability of the LDV-containing peptide to inhibit adhesion strengthening. Consequently, we questioned whether soluble LDV peptide would inhibit adhesion strengthening of the activation-defective Jurkat variants. As shown in Fig. 6*C*, adhesion of the activation-defective and wild-type Jurkat cells on VCAM-1 was equally inhibited by LDV peptide. This suggests that while the activation-defective Jurkat cells have a reduced affinity for sVCAM-1, their binding affinity for soluble LDV peptide is unaffected. Collectively, these results suggest that loss of $\alpha_4\beta_1$ activation in these Jurkat variants has little effect on cell adhesion to VCAM-1 under static or flow conditions.

Discussion

Previous work established that cells can actively regulate the affinity of integrin $\alpha_4\beta_1$ for VCAM-1 (activation) (8). In the present study, we have assessed the biological role of $\alpha_4\beta_1$ activation by

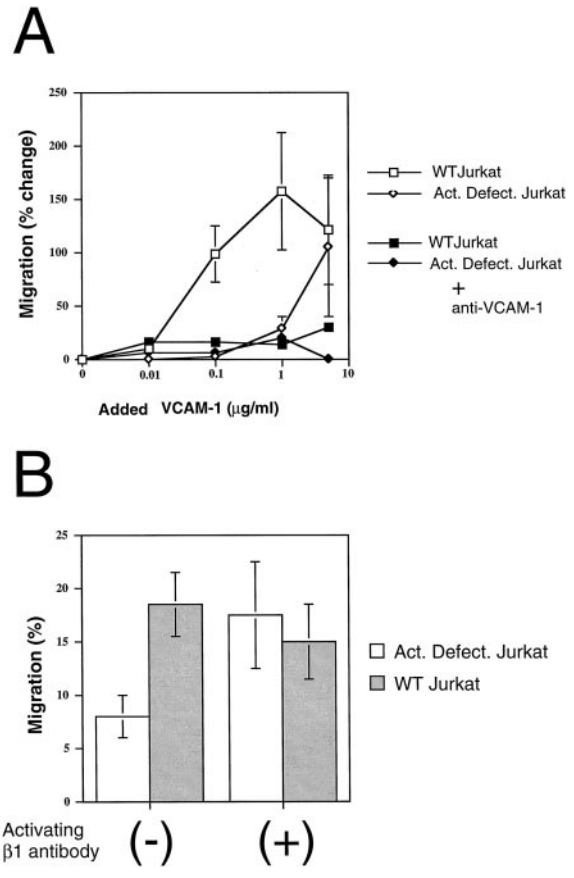

FIGURE 3. Loss of $\alpha_4\beta_1$ integrin activation causes decreased sensitivity to VCAM-1-stimulated β_2 integrin-dependent migration. *A*, Migration of wild-type and $\alpha_4\beta_1$ activation-defective Jurkat T cell variants. Transwell membranes were coated with 200 μg/ml ICAM-1 and indicated concentrations of VCAM-1. Migration assays were performed as described in Fig. 2 legend. *B*, Activating β_1 Ab rescues activation-defective Jurkat T cell migration. Migration assays were performed as in *A*, but cells were either untreated or treated with mAb 8A2 (0.1 μg/ml). Results are means ± SEM of three separate experiments.

deriving Jurkat T cell lines that lack high-affinity VCAM-1 binding. Using these cell lines, we observed that loss of $\alpha_4\beta_1$ activation dramatically reduces the sensitivity of cells to VCAM-1-stimulated $\alpha_L\beta_2$-dependent cell migration. In contrast, defective $\alpha_4\beta_1$ activation enhanced migration on purified VCAM-1 substrates, alone, and had little impact on static cell adhesion to VCAM-1 or

Table I. *Migration of $\alpha_4\beta_1$ activation defective Jurkat T cell variants*[a]

Substrate	Migration (%)			
	Jurkat	JA1	JA3	JD6
ICAM-1	10.2	11.0	8.4	10.5
VCAM-1	16.4	28.0	22.6	26.6
ICAM-1 + VCAM-1	22.2	14.0	11.9	13.3

[a] Representative migration results for three independently derived $\alpha_4\beta_1$ activation defective Jurkat variants. Migration assay was performed as described in *Materials and Methods.* Transwell membranes were coated with either ICAM-1 (10 μg/ml) or VCAM-1 (30 μg/ml) alone or a combination of ICAM-1 (200 μg/ml) and VCAM-1 (1 μg/ml). Results are expressed a percentage of input cells.

on adhesion in shear flow. These results lead us to propose that the activation of integrin $\alpha_4\beta_1$ is involved in the specification of preferred anatomical sites of leukocyte transmigration from the vasculature.

To study the functional role of $\alpha_4\beta_1$ affinity modulation, we used chemical mutagenesis and flow cytometry to isolate Jurkat lines with defects in $\alpha_4\beta_1$ activation. Using a VCAM-Ig fusion protein as a soluble ligand for $\alpha_4\beta_1$, we previously established that ~20% of $\alpha_4\beta_1$ expressed on Jurkat cells is capable of binding sVCAM-1 with high affinity (EC_{50} ~50 nM) (8), whereas <1% of the $\alpha_4\beta_1$ was activated on the defective Jurkat cells. The defect in sVCAM-1 binding in these mutant lines was not due to a reduction in integrin expression, as the mutant lines expressed wild-type levels of $\alpha_4\beta_1$. Furthermore, the loss of sVCAM-1 binding was not due to an intrinsic defect in the ligand binding site as sVCAM-1 binding could be rescued with exogenous integrin activators such as Mn^{2+} or activating Abs. The defect was not ascribable to an integrin mutation, because overexpression of wild-type α_4 and/or β_1 subunits failed to rescue sVCAM-1 binding. These results indicate that an element(s) of the intrinsic signaling pathway required for $\alpha_4\beta_1$ activation in these cells was disrupted. These $\alpha_4\beta_1$ activation-defective cells provide a tool to study the role of $\alpha_4\beta_1$ affinity modulation on T cell function.

$\alpha_4\beta_1$ affinity modulation is involved in regulating α_4 signaling to β_2 integrins. α_4 integrins can function as signaling receptors as well as adhesion receptors. α_4 integrin engagement influences biochemical pathways that affect cell function such as metalloproteinase gene expression (18). α_4 integrin signaling can also affect the function of other integrin family members, a process termed *trans* activation/suppression or cross-talk (19, 20). $\alpha_4\beta_1$ signaling stimulates β_2-dependent cell adhesion (10, 11) and, as confirmed in the present work, β_2-dependent cell migration. Coimmobilization of

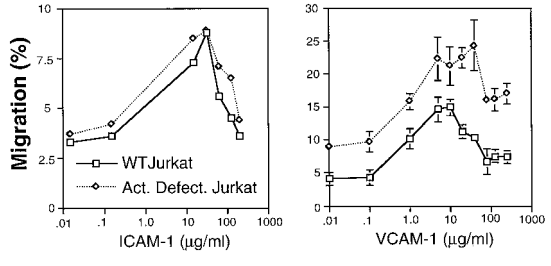

FIGURE 4. Migration of wild-type and activation-defective Jurkat variants on ICAM-1 or VCAM-1. Transwell membranes were coated with the indicated concentrations of either ICAM-1 (*left panel*) or VCAM-1 (*right panel*). Wild-type (□) and $\alpha_4\beta_1$ activation-defective (◇) Jurkat T cell variants were allowed to migrate for 4 h at 37°C using SDF-1α (15 ng/ml) as a chemoattractant. Cells migrating to the bottom chamber were enumerated with a hemocytometer, and migration was expressed as a percentage of input cells. Results are mean ± SEM of three separate experiments.

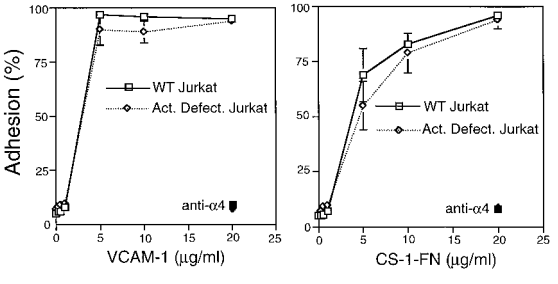

FIGURE 5. Static adhesion of wild-type and $\alpha_4\beta_1$ activation-defective Jurkat variants. Plates were coated with the indicated concentrations of either VCAM-1 (*left panel*) or CS-1 fragment of fibronectin (*right panel*). Wild-type Jurkat (□) or $\alpha_4\beta_1$ activation-defective Jurkat variants (◇) alone or with the addition of anti-α_4 Ab (HP2/1) (20 μg/ml) were allowed to adhere for 40 min at 37°C. Adhesion was quantified by crystal violet staining and expressed as a percentage of input cells. Results are mean ± SEM of three separate experiments.

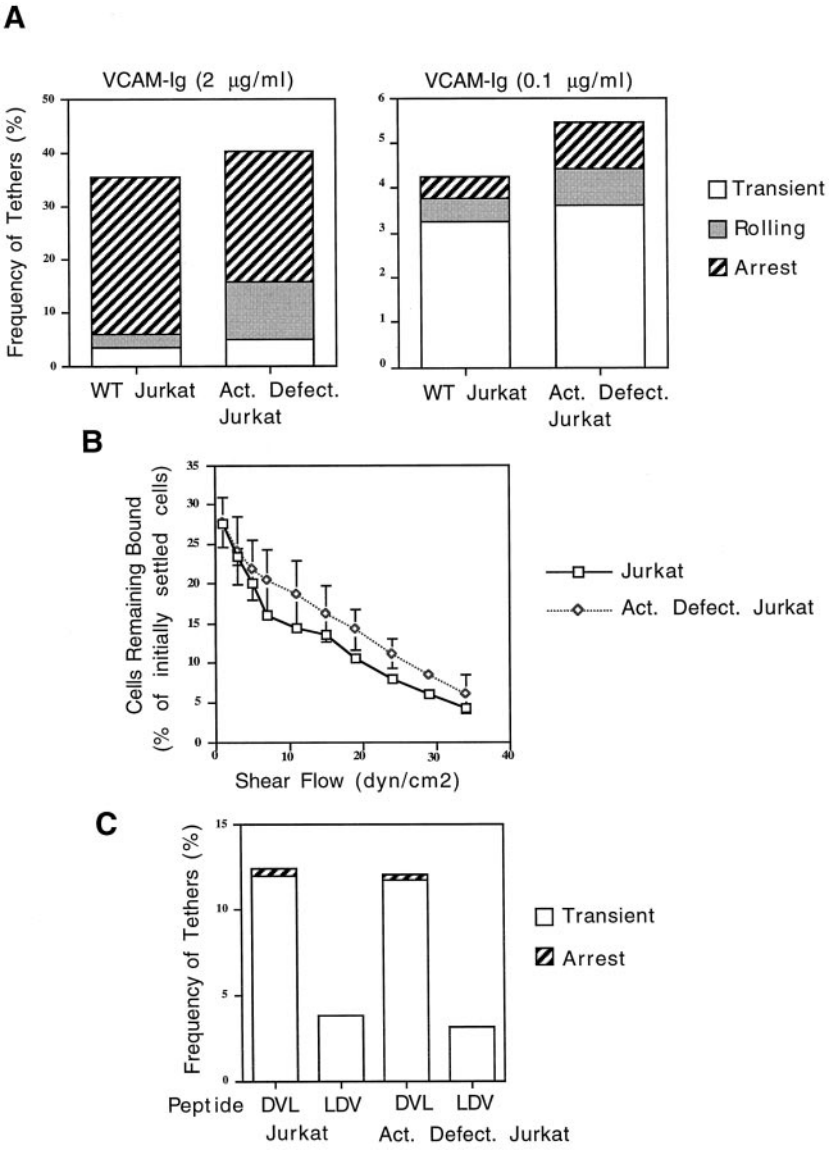

FIGURE 6. Adhesion of wild-type and $\alpha_4\beta_1$ activation-defective Jurkat variants under flow conditions. *A*, Cells were analyzed at a shear stress of 1 dyne/cm^2 on a VCAM-1 (soluble monovalent seven-domain VCAM-1) coated at 1 or 0.2 μg/ml. Cells were monitored for 10 s after the initial tethering event and were grouped into cells either transiently tethered, tethered and rolling, or arresting immediately after tethering to the adhesive substrates (see *Materials and Methods*). The fraction of cells (of the cell flux coming in close contact with the substrate) is presented in frequency units in a stacked bar graph. *B*, Resistance to detachment by shear flow. Cells were settled for 1 min on VCAM-1 at a coating of 0.1 μg/ml under static conditions. Shear stress was increased stepwise every 5 s. The number of cells that remained bound was expressed relative to the original number settled on the VCAM-1-coated substrate. *C*, Effect of LDV octapeptide and DVL octapeptide control on $\alpha_4\beta_1$-mediated tethering of Jurkat and activation-defective Jurkat variant to low-density VCAM-1. Tethering frequencies were determined at a shear stress of 1 dyne/cm^2 in binding medium containing 0.5 mM of either peptide. Tethering categories (transient or arrest) are depicted, respectively, in the filled and open bars. Data depicted are representative of three independent flow experiments.

VCAM-1, as a ligand for $\alpha_4\beta_1$, and ICAM-1, as a ligand for β_2 integrins, stimulated Jurkat cell migration. The stimulated cell migration was mediated by β_2 integrins, as the increased migration was blocked with an anti-β_2 Ab. The stimulated migration was also blocked with an anti-VCAM-1 Ab, indicating that engagement of $\alpha_4\beta_1$ with ligand was required for the stimulated migration. Consequently, $\alpha_4\beta_1$ functions as a VCAM-1 receptor to stimulate β_2 integrin-dependent cell migration. In contrast to wild-type Jurkat cells, the activation-defective Jurkat cells required 20-fold more VCAM-1 to stimulate β_2-dependent migration. The defect in $\alpha_4\beta_1$ signaling to β_2 integrins in these Jurkat variants was at the level of VCAM-1 binding, because the rescue of high-affinity $\alpha_4\beta_1$ by an activating β_1 Ab also rescued the $\alpha_4\beta_1$-stimulated β_2-dependent cell migration. Thus, $\alpha_4\beta_1$ affinity, in conjunction with VCAM-1 ligand density, regulates the migratory function of β_2 integrins.

$\alpha_4\beta_1$ activation plays a minor role in the control of Jurkat cell adhesion under static and flow conditions. The wild-type and activation-defective Jurkat lines manifested similar adhesion to VCAM-1 and the CS-1 fragment of fibronectin under static conditions. Thus, the high-affinity $\alpha_4\beta_1$ on Jurkat cells do not appear to play a major role in regulating static cell adhesion. This finding is not unique to $\alpha_4\beta_1$, as integrin $\alpha_5\beta_1$ activation also plays only a minor role in static cell adhesion (21). Furthermore, wild-type and $\alpha_4\beta_1$ activation-defective Jurkat cells showed similar tethering/rolling and adhesion strengthening on VCAM-1 under flowing conditions. These results are in agreement with Yauch et al. (22), who reported that $\alpha_4\beta_1$ affinity modulation plays a minor role in adhesion. However, one study found that Jurkat variants with defective $\alpha_4\beta$-dependent static adhesion also had a defect in adhesion strengthening under flow (15). In that same study, a small LDV-containing peptide was found to inhibit adhesion strengthening of cells on VCAM-1. This led to the proposal that high-affinity $\alpha_4\beta_1$ was required for adhesion strengthening. It was surprising to find no difference in adhesion strengthening between our $\alpha_4\beta_1$ activation-defective and wild-type Jurkat cells. Furthermore, the soluble LDV peptide inhibited equally well in these two cell types. It is possible that our Jurkat variants may have lost high affinity for VCAM-1, but retained a high-affinity recognition of LDV peptide. Another possibility is that our sVCAM-1-binding mutants may have a specific defect in recognition of Ig domain 4 of VCAM-1. VCAM-1 contains two $\alpha_4\beta_1$ binding sites, Ig domains 1 and 4. It may be that soluble binding of VCAM-1 requires recognition of both domains 1 and 4. A loss of domain 4 recognition could interfere with sVCAM-1 binding and cell signaling without interfering with tethering under flow, which has been

found to be dependent on the VCAM-1 Ig domain 1. We are currently testing such a possibility with the use of specific mutants and Abs to VCAM-1 Ig domains 1 and 4.

Integrin activation is one mechanism for regulating integrin function (23). The biologic role of integrin affinity modulation is integrin and cell type specific (24). Activation of $\alpha_{IIb}\beta_3$ is critical for platelet aggregation, but it has little impact on platelet adhesion to fibrinogen (25). Similarly, activation of integrin $\alpha_5\beta_1$ appears to play a minor role in static cell adhesion, but is important in fibronectin matrix assembly (26, 27). The results reported in this work show little effect of $\alpha_4\beta_1$ activation on static adhesion. However, they define a new function for integrin activation: regulation of the sensitivity or threshold of leukocytes to stimulation by immobilized integrin ligands, such as VCAM-1.

Leukocyte subpopulations show tissue-specific trafficking patterns that govern immune responses (2, 3). These tropisms are dependent, in part, on adhesion molecule expression on leukocytes and vascular endothelium (5). Integrin abundance on leukocytes and counter ligand density on vascular endothelial cells is one proposed means to regulate leukocyte trafficking (28). For example, a subset of memory T cells, expressing high levels of $\alpha_4\beta_1$, migrates preferentially to nongastrointestinal, extralymphoid inflammatory sites, in which VCAM-1 is expressed (28–30). Our results suggest that $\alpha_4\beta_1$ activation may play an important role in determining leukocyte responses to different levels of VCAM-1. The affinity of $\alpha_4\beta_1$ acts as a set point for the cells' response to VCAM-1 and subsequent stimulation of β_2-dependent migration. Primary circulating leukocytes express varying levels of high-affinity $\alpha_4\beta_1$ (8), and VCAM-1 expression changes several-fold on blood vessels during the course of inflammation (13, 31). Consequently, the activation of $\alpha_4\beta_1$ may specify preferential recruitment of leukocyte subsets to sites of differing VCAM-1 densities.

Acknowledgments

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References

1. Shimizu, Y., D. M. Rose, and M. H. Ginsberg. 1999. Integrins in the immune system. *Adv. Immunol. 72:325.*
2. Butcher, E. C., and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science 272:60.*
3. Butcher, E. C., M. Williams, K. Youngman, L. Rott, and M. Briskin. 1999. Lymphocyte trafficking and regional immunity. *Adv. Immunol. 72:209.*
4. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell 76:301.*
5. Carlos, T. M., and J. M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood 84:2068.*
6. Faull, R. J., and M. H. Ginsberg. 1996. Inside-out signaling through integrins. *J. Am. Soc. Nephrol. 7/8:1091.*
7. Bazzoni, G., and M. E. Hemler. 1998. Are changes in integrin affinity and conformation overemphasized? *Trends Biochem. Sci. 23:30.*
8. Rose, D. M., P. M. Cardarelli, R. R. Cobb, and M. H. Ginsberg. 2000. Soluble VCAM-1 binding to $\alpha_4\beta_1$ integrins is cell-type specific and activation dependent and is disrupted during apoptosis in T cells. *Blood 95:602.*
9. Kassner, P. D., R. Alon, and T. A. Springer. 1995. Specialized functional properties of the integrin α_4 cytoplasmic domain. *Mol. Biol. Cell 6:661.*
10. Chan, J., S. Hyduk, and M. I. Cybulsky. 2000. $\alpha_4\beta_1$ integrin/VCAM-1 integration activates $\alpha_L\beta_2$ integrin-mediated adhesion to ICAM-1 in human T cells. *J. Immunol. 164:746.*
11. May, A. E., F. J. Neumann, A. Schomig, and K. T. Preissner. 2000. VLA-4 engagement defines a novel activation pathway for β_2 integrin-dependent leukocyte adhesion involving the urokinase receptor. *Blood 96:506.*
12. Bevilacqua, M. P. 1993. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol. 11:767.*
13. Henninger, D. D., J. Panes, M. Eppihimer, J. Russell, M. Gerritsen, D. C. Anderson, and D. N. Granger. 1997. Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. *J. Immunol. 158:1825.*
14. Jongewaard, I. N., P. M. Tsai, and J. W. Smith. 1996. The type III connecting segment of fibronectin contains an aspartic acid residue that regulates the rate of binding of integrin $\alpha_4\beta_1$. *Cell Adhes. Commun. 3:487.*
15. Chen, C., J. L. Mobley, O. Dwir, F. Shimron, V. Grabovsky, R. R. Lobb, Y. Shimizu, and R. Alon. 1999. High-affinity very late antigen-4 subsets expressed on T cells are mandatory for spontaneous adhesion strengthening but not rolling on VCAM-1 in shear flow. *J. Immunol. 162:1084.*
16. Weber, C., and T. A. Springer. 1998. Interaction of very late antigen-4 with VCAM-1 supports transendothelial chemotaxis of monocytes by facilitating lateral migration. *J. Immunol. 161:6825.*
17. Berlin, C., R. F. Bargatze, J. J. Campbell, U. H. von Andrian, M. C. Szabo, S. R. Hasslen, R. D. Nelson, E. L. Berg, S. L. Erlandsen, and E. C. Butcher. 1995. α_4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell 80:413.*
18. Romanic, A. M., and J. A. Madri. 1994. The induction of 72-kD gelatinase in T cells upon adhesion to endothelial cells is VCAM-1 dependent. *J. Biol. Chem. 125:1165.*
19. Porter, J. C., and N. Hogg. 1997. Integrin cross talk: activation of lymphocyte function-associated antigen-1 on human T cells alters $\alpha_4\beta_1$- and $\alpha_5\beta_1$-mediated function. *J. Cell Biol. 138:1437.*
20. Porter, J. C., and N. Hogg. 1998. Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol. 8:390.*
21. Faull, R. J., N. L. Kovach, J. M. Harlan, and M. H. Ginsberg. 1994. Stimulation of integrin-mediated adhesion of T lymphocytes and monocytes: two mechanisms with divergent biological consequences. *J. Exp. Med. 179:1307.*
22. Yauch, R. L., D. P. Felsenfeld, S. K. Kraeft, L. B. Chen, M. P. Sheetz, and M. E. Hemler. 1997. Mutational evidence for control of cell adhesion through integrin diffusion/clustering, independent of ligand binding. *J. Exp. Med. 186:1347.*
23. Hughes, P. E., and M. Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol. 8:359.*
24. O'Toole, T. E., Y. Katagiri, R. J. Faull, K. Peter, R. N. Tamura, V. Quaranta, J. C. Loftus, S. J. Shattil, and M. H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol. 124:1047.*
25. Shattil, S. J., H. Kashiwagi, and N. Pampori. 1998. Integrin signaling: the platelet paradigm. *Blood 91:2645.*
26. Wu, C., V. M. Keivens, T. E. O'Toole, J. A. McDonald, and M. H. Ginsberg. 1995. Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. *Cell 83:715.*
27. Sechler, J. L., S. A. Corbett, and J. E. Schwarzbauer. 1997. Modulatory roles for integrin activation and the synergy site of fibronectin during matrix assembly. *Mol. Biol. Cell 8:2563.*
28. Volpes, R., J. J. van den Oord, and V. J. Desmet. 1991. Memory T cells represent the predominant lymphocyte subset in acute and chronic liver inflammation. *Hepatology 13:826.*
29. Horgan, K. J., G. E. Luce, Y. Tanaka, T. Schweighoffer, Y. Shimizu, S. O. Sharrow, and S. Shaw. 1992. Differential expression of VLA-α_4 and VLA-β_1 discriminates multiple subsets of $CD4^+CD45RO^+$ "memory" T cells. *J. Immunol. 149:4082.*
30. Engelhardt, B., F. C. Conley, P. J. Kilshaw, and E. C. Butcher. 1995. Lymphocytes inflitrating the CNS during inflammation display a distinctive phenotype and bind VCAM-1 but not MAdCAM-1. *Int. Immunol. 7:481.*
31. Langley, R. R., J. Russell, M. J. Eppihimer, S. J. Alexander, M. Gerritsen, R. D. Specian, and D. N. Granger. 1999. Quantification of murine endothelial cell adhesion molecules in solid tumors. *Am. J. Physiol. 277:H1156.*