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$\alpha_4\beta_1$ Integrin/VCAM-1 Interaction Activates $\alpha_L\beta_2$ Integrin-Mediated Adhesion to ICAM-1 in Human T Cells[1]

Jason R. Chan,[2] Sharon J. Hyduk,[2] and Myron I. Cybulsky[3]

Modulation of integrin affinity and/or avidity provides a regulatory mechanism by which leukocyte adhesion to endothelium is strengthened or weakened at different stages of emigration. In this study, we demonstrate that binding of high-affinity $\alpha_4\beta_1$ integrins to VCAM-1 strengthens $\alpha_L\beta_2$ integrin-mediated adhesion. The strength of adhesion of Jurkat cells, a human leukemia T cell line, or $MnCl_2$-treated peripheral blood T cells to immobilized chimeric human VCAM-1/Fc, ICAM-1/Fc, or both was quantified using parallel plate flow chamber leukocyte detachment assays in which shear stress was increased incrementally (0.5–30 dynes/cm^2). The strength of adhesion to VCAM-1 plus ICAM-1, or to a 40-kDa fragment of fibronectin containing the CS-1 exon plus ICAM-1, was greater than the sum of adhesion to each molecule alone. Treatment of Jurkat or blood T cells with soluble cross-linked VCAM-1/Fc or HP2/1, a mAb to α_4, significantly increased adhesion to ICAM-1. These treatments induced clustering of $\alpha_L\beta_2$ integrins, but not the high-affinity β_2 integrin epitope recognized by mAb 24. Up-regulated adhesion to ICAM-1 was abolished by cytochalasin D, an inhibitor of cytoskeletal rearrangement. Taken together, our data suggest that the binding of VCAM-1 or fibronectin to $\alpha_4\beta_1$ integrins initiates a signaling pathway that increases β_2 integrin avidity but not affinity. A role for the cytoskeleton is implicated in this process. *The Journal of Immunology,* **2000, 164: 746–753.**

Integrins are cell adhesion and signaling molecules with complex biologic features, including regulated ligand-binding capabilities and interactions with multiple intracellular cytoskeletal and signaling proteins. Cell-cell or cell-matrix contacts generate integrin-mediated signals that are necessary for cell growth, survival, or locomotion. Integrins consist of α and β transmembrane protein subunits associated noncovalently. At least 16 α and 8 β subunits have been described, accounting for over 20 heterodimeric combinations with unique cell-type distribution patterns and diverse ligand binding characteristics (1–3).

Mononuclear leukocytes express α_4 and β_2 integrins. On T lymphocytes, the major integrins are $\alpha_4\beta_1$ (VLA-4, CD49d/CD29) and $\alpha_L\beta_2$ (LFA-1, CD11a/CD18), which bind to VCAM-1 (CD106) and ICAM-1 (CD54), respectively (4, 5). These integrins participate in all aspects of leukocyte biology and each plays a critical role at specific stages of leukocyte emigration from blood into tissues. Emigration is a multistep process involving sequential leukocyte-endothelial adhesive interactions that include tethering, rolling, stable adhesion, spreading, and migration across the endothelium into extravascular tissues (6). Both α_4 and β_2 integrins can mediate stable adhesion, leukocyte spreading, and migration, whereas only α_4 integrins can mediate initial rolling interactions (7, 8).

On circulating leukocytes, constitutively expressed $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins have low ligand-binding capacity; however, at sites of emigration during inflammation or trafficking, these integrins are "activated" in a highly regulated manner. The process by which extracellular stimuli modulate integrin adhesive function has been referred to as inside-out signaling. Inside-out signals up-regulate integrin ligand binding capacity by increasing integrin affinity and/or avidity in response to stimulation by a wide array of cellular receptors. Alterations in integrin affinity are due to conformational changes that allow more efficient ligand binding. Epitopes unique to high-affinity β_1 or β_2 integrins can be detected with mAbs. The divalent cations Mn^{2+} or Mg^{2+} are extensively used as an exogenous mechanism for increasing the affinity of α_4 and β_2 integrins for their respective soluble ligands (9, 10). Intracellular signals that regulate both $\alpha_4\beta_1$ and β_2 integrin affinity on T cells are generated by ligation of L-selectin (11, 12). In contrast, the affinity of β_2 integrins but not β_1 integrins is up-regulated by CC chemokine stimulation of eosinophils (13). Avidity changes, which include integrin clustering, association with the cell cytoskeleton, and activation-dependent cell spreading, up-regulate integrin ligand binding without affecting affinity. Phorbol ester treatment of T cells triggers β_1 and β_2 integrin-mediated adhesion and cell spreading in the absence of changes in integrin affinity (14, 15). Similarly, cross-linking the TCR complex induces cell flattening, association of integrins with cytoskeletal proteins, and adhesion to VCAM-1 and ICAM-1 without altering integrin affinity (15–17). Although affinity and avidity are distinct processes, it is likely that both are involved in leukocyte emigration in vivo.

Ligand binding by integrins can result in the generation of diverse intracellular signals. This outside-in signaling is instrumental to various cell processes. For example, cross-linking of β_2 integrins can prime neutrophils for cytokine-induced oxidative burst (18), and monocyte adherence to fibronectin or engagement of $\alpha_4\beta_1$ has been demonstrated to stimulate production of potent inflammatory mediators such as TNF-α, IL-1, and the procoagulant tissue factor protein (19–21). Outside-in signals can also modulate the function of other integrins by initiating an inside-out signal,

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and this "cross-talk" between integrins may be important for leukocyte emigration. For example, binding of $\alpha_L\beta_2$ integrins to ICAM-1 decreased the ability of $\alpha_4\beta_1$ integrins to bind fibronectin through an avidity-dependent mechanism (22). This may have implications for the progression of T lymphocytes from stable adhesion to transmigration.

The present study seeks to determine whether cross-talk exists between $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins at an earlier stage of leukocyte emigration. It is known that binding of GlyCAM-1 to L-selectin or soluble P-selectin to P-selectin glycoprotein ligand-1 up-regulates leukocyte integrin function, which results in strengthening of adhesion (11, 12, 23). Like the selectins, $\alpha_4\beta_1$ integrins can mediate T lymphocyte rolling. Therefore, we postulated that $\alpha_4\beta_1$ binding to VCAM-1 may also strengthen $\alpha_L\beta_2$-mediated adhesion of T lymphocytes to ICAM-1. Accordingly, we used a parallel plate flow chamber leukocyte detachment assay to monitor the strength of T lymphocyte adhesion to immobilized VCAM-1 and ICAM-1. Our data suggest that VCAM-1 binding to $\alpha_4\beta_1$ integrin results in increased β_2 integrin-mediated adhesion of T lymphocytes to ICAM-1 via an avidity-dependent mechanism.

Materials and Methods

Reagents

Chimeric VCAM-1 (VCAM-1/Fc)[4] and ICAM-1 (ICAM-1/Fc) were a gift from Dr. Donald Staunton (ICOS Corporation, Bothwell, WA) and have been previously characterized (24). VCAM-1/Fc and ICAM-1/Fc consisted of the seven and five extracellular Ig-like domains of VCAM-1 and ICAM-1, respectively, linked to the Fc fragment of human IgG1, containing the hinge region, CH2 and CH3 domains. The following mAbs were acquired: HP2/1 to human CD49d (α_4 subunit) from Serotec (Oxford, U.K.), TS1/22 to human CD11a (α_L subunit) from Endogen (Woburn, MA), and mAb 24, an activation reporter for β_2 integrins (25), from Dr. Nancy Hogg (Imperial Cancer Research Fund, U.K.). Goat anti-human IgG (Fc specific) F(ab′)$_2$ were obtained from Caltag (Burlingame, CA). Secondary Abs for flow cytometry and confocal microscopy included Cy3-conjugated donkey anti-mouse IgG, FITC-conjugated donkey anti-goat IgG, and FITC-conjugated goat anti-mouse IgG, all obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Other reagents included the 40-kDa fragment of fibronectin (FN40) (Life Technologies, Gaithersburg, MD), cytochalasin D (Calbiochem, La Jolla, CA), Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA), and PMA, manganese chloride, magnesium chloride, EGTA, EDTA, nonimmune human IgG1, and 0.01% poly-L-lysine (Sigma, St. Louis, MO).

Cell culture and T cell isolation

Jurkat E6-1, a human TCR $\alpha\beta^+$, CD2$^+$, CD3$^+$, CD4$^+$, CD8$^-$, CD28$^+$ T cell line derived from an EBV-negative, non-Hodgkins lymphoblastic leukemia (26), was obtained from American Type Culture Collection (Rockville, MD). Jurkat cells are a useful model for studying integrin regulation because many of the signaling events thought to be important in integrin function are intact in this cell line (27). Leukocyte integrins expressed on Jurkat cells include $\alpha_4\beta_1$ but not $\alpha_4\beta_7$ (28) and $\alpha_L\beta_2$ but not $\alpha_M\beta_2$ (data not shown). Jurkat cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies) and 100 U/ml penicillin-streptomycin (Life Technologies).

Human peripheral blood T cells were isolated by density gradient centrifugation and Ab/magnetic particle depletion. Venous blood (30 ml) was obtained from healthy normal volunteers and collected in EDTA (3 mmol/L final concentration). Buffy coat leukocytes were layered over Histopaque 1077 (Sigma) and centrifuged at 300 × *g* to obtain mononuclear leukocytes. T cells were purified by negative depletion of monocytes, B lymphocytes, NK cells, and contaminating granulocytes with a kit from Miltenyi Biotec (Auburn, CA) using specific Abs and magnetic particles according to the manufacturer's protocol. Purified T cells were resuspended in RPMI 1640, 1% FBS, and kept at 10°C. Just before experiments, cells were centrifuged and resuspended in the assay buffer.

[4] Abbreviations used in this paper: VCAM-1/Fc, recombinant human fusion protein VCAM-1 and Fc portion of IgG; ICAM-1/Fc, recombinant human fusion protein ICAM-1 and Fc portion of IgG; FN40, 40-kDa fragment of fibronectin; CS-1, connecting segment-1.

Immobilization of VCAM-1/Fc, ICAM-1/Fc, and FN40

VCAM-1/Fc and ICAM-1/Fc were immobilized on plastic by the following procedure. Goat anti-human IgG (Fc specific) F(ab′)$_2$ was passively adsorbed onto the center of a 35-mm polystyrene tissue culture dish by incubating a 10-μl drop (100 μg/ml) for 60 min in a humidified atmosphere (22°C). Dishes were washed with PBS and nonspecific binding sites were blocked with 5% FBS (60 min, 22°C). Scatchard analysis using I^{125} radiolabeled human IgG1 (Lofstrand Labs, Gaithersburg, MD) indicated that the anti-Fc-coated surface supported a maximum of 1200 IgG1 molecules per μm^2 (J.R.C., unpublished data). The anti-Fc-coated area was incubated with a saturating concentration (20 μg/ml in PBS) of VCAM-1/Fc, ICAM-1/Fc, and/or nonimmune human IgG1 (10-μl drop, 60 min, 22°C). The coating density of each molecule was regulated by adjusting the relative molarity. Control plates were incubated with nonimmune human IgG1 only.

The above procedure was modified for immobilization of FN40. After passive adsorption of goat anti-human IgG (Fc specific) F(ab′)$_2$, dishes were incubated with 10 μl of FN40 (250 μg/ml, 60 min, 22°C) before blocking of nonspecific binding sites with 5% FBS. For FN40-coated dishes without ICAM-1 coimmobilization, anti-human IgG (Fc specific) binding sites were occupied with nonimmune human IgG.

Detachment assay

Cell detachment assays were performed using a parallel plate flow chamber purchased from Glycotech (Rockville, MD). The adhesion molecule-coated 35-mm tissue culture dish served as the bottom surface and a silicone gasket formed a 0.254-mm (0.010 inch) high and 2.5-mm wide flow path. Leukocytes suspended in assay buffer (1.5×10^6/ml) were injected via the outflow port into inverted flow chambers. Chambers were then overturned and leukocytes settled for 10 min under static conditions onto the adhesion molecule-coated surface. Shear stress was applied by pulling assay buffer through chambers with a Genie programmable syringe pump (Kent Scientific, Litchfield, CT) starting at 0.5 dynes/cm^2 for 120 s, then increasing to 30 dynes/cm^2 at 30-s intervals. Wall shear stress (τ, dynes/cm^2) was determined using the formula $\tau = 6Q\mu/bh$ (2), where Q is flow rate (ml/s), μ is viscosity (0.01 dyne · s/cm^2), b is channel width (cm), and h is channel height (cm). Cells were observed with a Diaphot 300 inverted phase contrast microscope (Nikon, Melville, NY) and videotaped with a Sony DXC-151A color video camera and Sony SVT-S3100 time lapse video cassette recorder. The assay buffer was kept in a 37°C water bath, and the temperature of the flow chambers was maintained at 37°C using an infrared heat lamp, a thermocouple probe in the outflow tube, and a temperature controller (CN76000; Omega Engineering, Laval, Canada). The number of cells attached before the introduction of shear stress (80–120) and after each shear stress interval was determined from video tape frames and expressed as a percentage of cells remaining attached.

Standard assay buffer consisted of HBSS (1 mmol/L Mg^{2+} and 1 mmol/L Ca^{2+}) with 20 mmol/L HEPES. For some experiments, the divalent cation content was varied (1 mmol/L Mg^{2+}, 1 mmol/L Ca^{2+}, and 1 mmol/L Mn^{2+} or 5 mmol/L Mg^{2+} and 1 mmol/L EGTA) or reagents to cross-link $\alpha_4\beta_1$ integrins, PMA (50 nmol/L), and/or cytochalasin D (2 μg/ml) were added. Leukocytes were incubated in assay buffer for 15 min (37°C) before injection into the flow chamber. In Ab blocking experiments, leukocytes were preincubated with mAbs HP2/1, TS1/22, or both (10 μg/ml, 30 min, 4°C).

Cross-linking of leukocyte α_4 integrins

Two different protocols were used to cross-link leukocyte $\alpha_4\beta_1$ integrins. Soluble cross-linked VCAM-1/Fc complexes were prepared by overnight incubation at 4°C of VCAM-1/Fc (1 μg/ml) with goat anti-human IgG F(ab′)$_2$ (Fc specific) (2 μg/ml). Nonimmune IgG (1 μg/ml) was used to make control complexes. Leukocytes were also incubated with mAb HP2/1 for 30 min on ice followed by cross-linking with goat anti-mouse IgG for 15 min at 37°C.

Flow cytometry

Monoclonal Abs were used in flow cytometry experiments to analyze the expression of $\alpha_L\beta_2$ integrins (TS1/22) and its affinity state (mAb 24). During treatment with soluble VCAM-1/Fc complexes, Mg/EGTA, or PMA, leukocytes were incubated with one of the above primary Abs (10 μg/ml, 20 min, 37°C), followed by secondary Ab (Cy3-conjugated donkey anti-mouse IgG, 1:100 dilution, 30 min, on ice). Leukocytes were washed after primary and secondary Ab incubations with assay buffer. Binding of soluble VCAM-1/Fc complexes was detected with FITC-conjugated donkey anti-goat IgG (1:100 dilution, 30 min, on ice). Flow cytometry was conducted on all samples immediately after the final wash using an Epics

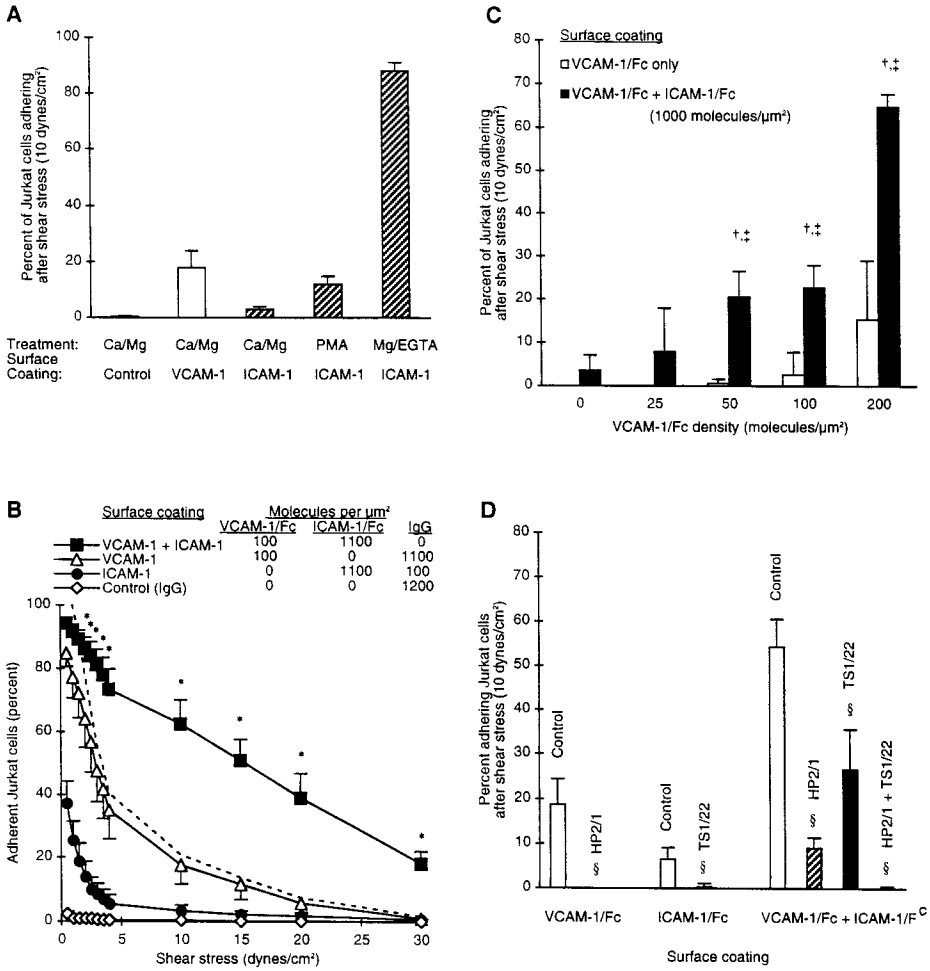

FIGURE 1. Synergistic adhesion of Jurkat cells to coimmobilized VCAM-1 and ICAM-1. *A*, Jurkat cell adhesion to control (nonimmune human IgG), VCAM-1-, and ICAM-1-coated surfaces (coating densities same as *B*). The assay buffer contained 1 mmol/L of Ca^{2+} and Mg^{2+} (Ca/Mg), and Jurkat cells were treated with PMA (50 nmol/L) or 5 mmol/L Mg^{2+}/1 mmol/L EGTA (Mg/EGTA). Mean ± SEM of cell remaining adherent after exposure to a shear stress of 10 dynes/cm^2 ($n = 5$). *B*, Jurkat cell adhesion to immobilized VCAM-1 + ICAM-1, VCAM-1, ICAM-1, or nonimmune IgG after stepwise increases in shear stress. The assay buffer contained 1 mmol/L of Ca^{2+} and Mg^{2+}. Mean ± SEM of 10–12 samples collected over four experimental days. ∗, Significant difference from VCAM-1, determined by ANOVA and Bonferroni-Dunn posthoc test ($p < 0.01$). *C*, Jurkat cell adhesion to various densities of VCAM-1 coimmobilized with or without ICAM-1 (1000 ICAM-1/Fc molecules/μm^2). After 10 min of static adhesion, cells were exposed to a shear stress of 10 dynes/cm^2. Mean ± SD of six nonoverlapping microscope fields. †, Significant difference from VCAM-1 alone, determined by Student's *t* test ($p < 0.05$). ‡, Significant difference from 0 VCAM-1/Fc and 1000 ICAM-1/Fc molecules/μm^2, determined by ANOVA and Bonferroni-Dunn posthoc test ($p < 0.01$). *D*, Monoclonal Ab blockade of Jurkat cell adhesion to VCAM-1, ICAM-1, or both adhesion molecules. The coating densities were the same as in *B*. Jurkat cells were incubated with HP2/1 and/or TS1/22 or buffer before 10 min of static adhesion and exposure to 10 dynes/cm^2 of fluid shear stress. The percentage of cells remaining adherent is shown. Mean ± SD for six high power fields. §, Significant difference compared with no mAb treatment by Student's *t* test ($p < 0.05$) or ANOVA and Bonferroni-Dunn posthoc test ($p < 0.01$), where applicable.

XL-MCL flow cytometer (Coulter, Miami, FL). Dead cells were eliminated from analyses by electronic gating of cells that failed to exclude propidium iodide (1 μg/ml).

Confocal microscopy

Jurkat cells (1×10^6/ml) were treated with soluble VCAM-1/Fc and cross-linking anti-human IgG F(ab′)$_2$ (Fc specific) or PMA (50 nmol/L) for 5 min (37°C) and allowed to adhere for an additional 15 min (37°C) to 0.01% poly-L-lysine-coated glass slides. Cells were then immediately placed on ice for 10 min followed by incubation with primary Abs (TS 1/22 for α_L integrin, W6/32 for class I MHC, or K16/16 nonbinding Ab) for 60 min. Nonadherent cells were washed off with assay buffer, and adherent cells were fixed with 0.5% paraformaldehyde for 15 min. Cy3-donkey anti-mouse IgG secondary Ab was added for 60 min on ice in the dark. Samples were mounted in Vectashield and observed at ×60 magnification (NA 1.4; Nikon) with a confocal microscope (MRC1024ES; Bio-Rad, Hercules, CA) using the following settings: magnification, 2.0; pinhole, 2.0 μm; and gain, 1000. Signals were specific because no fluorescence was detected with K16/16.

Results

Synergistic adhesion of Jurkat cells to coimmobilized VCAM-1 and ICAM-1

The objective of initial experiments was to determine whether binding of Jurkat cells to immobilized VCAM-1 strengthens adhesion to ICAM-1. The strength of Jurkat cell adhesion to immobilized VCAM-1/Fc, ICAM-1/Fc, or a combination of both molecules was compared using a parallel plate flow chamber leukocyte detachment assay. Jurkat cells adhered readily to VCAM-1/Fc (100 molecules/μm^2), but very weakly to ICAM-1/Fc even when ICAM-1/Fc was immobilized at a high density (1100 molecules/μm^2) (Fig. 1). Firm adhesion to ICAM-1/Fc could be observed only after treatments that increased either integrin affinity (Mg^{2+}/EGTA) or avidity (PMA) (Fig. 1*A*). These observations were consistent with previous reports (29) and indicate that Jurkat cells are

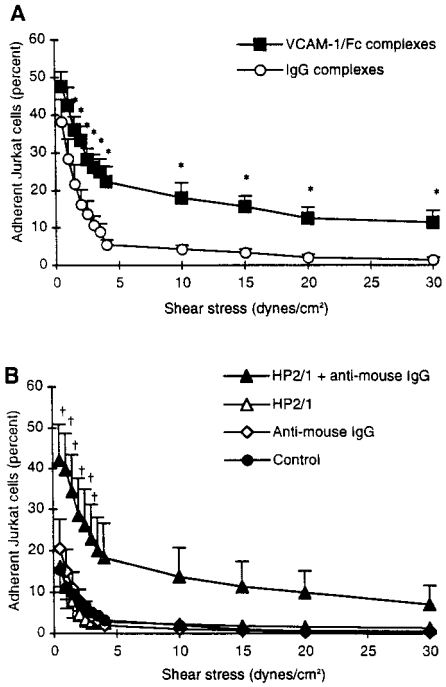

FIGURE 2. Cross-linking $\alpha_4\beta_1$ integrins strengthens Jurkat cell adhesion to ICAM-1. Before introduction into flow chambers with an ICAM-1/Fc-coated surface (1100 molecules/μm^2), Jurkat cells were treated with (*A*) soluble complexes of VCAM-1/Fc + anti-human IgG (VCAM-1/Fc complexes) or nonimmune human IgG + anti-human Ig (IgG complexes) or (*B*) various combinations of HP2/1 and/or an anti-mouse IgG Ab. Fluid shear stress (0–30 dynes/cm^2) was applied after 10 min of static adhesion and the percentage of cells remaining adherent at the end of each shear stress level is shown. Mean ± SEM of 6–10 samples collected over three experimental days. ∗, Significant difference, determined by Student's *t* test ($p < 0.05$). †, Significant difference from controls, determined by ANOVA and Bonferroni-Dunn posthoc test ($p < 0.01$).

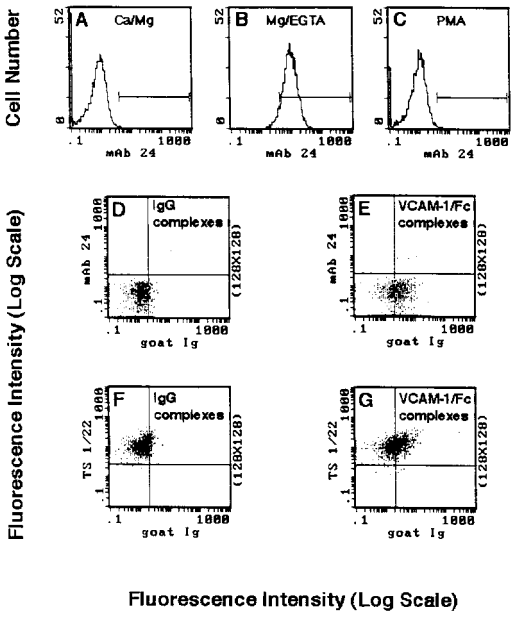

FIGURE 3. Cross-linking $\alpha_4\beta_1$ integrin does not affect β_2 integrin affinity. Flow cytometry analysis of Jurkat cells following treatment with (*A*) 1 mmol/L of Ca^{2+} and Mg^{2+} (Mg/Ca), (*B*) 5 mmol/L Mg^{2+}/1 mmol/L EGTA (Mg/EGTA), (*C*) PMA (50 nmol/L), (*D* and *F*) soluble nonimmune human IgG-goat anti-human IgG (IgG complexes), and (*E* and *G*) soluble VCAM-1/Fc-goat anti-human IgG (VCAM-1/Fc complexes). After treatments Jurkat cells were stained with mAb 24 (*A–C*), mAb 24 + anti-goat IgG (*D* and *E*), or TS 1/22 + anti-goat IgG (*F* and *G*). Treatments with PMA and VCAM-1/Fc complexes did not affect binding of mAb 24 or TS1/22. Data are representative of three individual experiments.

a model of T lymphocytes with "active" $\alpha_4\beta_1$, but "inactive" β_2 integrins. In experiments where VCAM-1/Fc and ICAM-1/Fc were coimmobilized, a low density of VCAM-1/Fc was chosen to support minimum Jurkat cell adhesion and allow maximum ICAM-1/Fc immobilization. Jurkat cell adhesion to both VCAM-1/Fc and ICAM-1/Fc was more than additive to either molecule alone (Fig. 1*B*), suggesting that binding of α_4 integrins to VCAM-1 induced intracellular signals that up-regulated $\alpha_L\beta_2$-mediated adhesion. This synergistic adhesion strengthening was evident for all tested concentrations of coimmobilized VCAM-1/Fc and ICAM-1/Fc (Fig. 1*C*). Jurkat cell adhesion was specific because preincubation with function blocking mAbs HP2/1 (anti-human α_4 integrin) and TS1/22 (anti-human α_L integrin) abolished adhesion to VCAM-1/Fc and ICAM-1/Fc, respectively. Preincubation with both HP2/1 and TS1/22 completely abolished Jurkat cell adhesion to coimmobilized VCAM-1/Fc and ICAM-1/Fc (Fig. 1*D*).

Cross-linking of $\alpha_4\beta_1$ integrins strengthens Jurkat cell adhesion to ICAM-1

Based on detachment assays with coimmobilized VCAM-1 and ICAM-1, it was difficult to ascertain which integrins were mediating cell signaling or increased adhesion. Therefore, α_4 integrins on Jurkat cells were cross-linked to initiate signaling before evaluating $\alpha_L\beta_2$-mediated adhesion to ICAM-1/Fc-coated surfaces. In these experiments, Jurkat cells were treated with either VCAM-1/Fc-goat anti-human IgG complexes or HP2/1, an anti-α_4 integrin mouse mAb, followed by cross-linking with goat anti-mouse IgG. Binding of soluble VCAM-1/Fc complexes and mAb HP2/1 to Jurkat cells was confirmed by indirect flow cytometry using FITC-conjugated donkey anti-human IgG and goat anti-mouse IgG, respectively. Both treatments resulted in strengthening of Jurkat cell adhesion to ICAM-1/Fc-coated surfaces (Fig. 2). In contrast, nonimmune human IgG complexes or non-cross-linked HP2/1 had no effect on adhesion. These data indicate that cross-linking of α_4 integrins by a physiological ligand or a function-blocking mAb strengthens $\alpha_L\beta_2$ integrin-mediated Jurkat cell adhesion to ICAM-1/Fc.

Cross-linking α_4 integrins does not alter β_2 integrin affinity

Binding of mAb 24, which recognizes an epitope that is unmasked only on β_2 integrins with high ligand-binding affinity (25), was used to determine whether cross-linking of α_4 integrins on Jurkat cells, as described above, resulted in increased β_2 integrin affinity. Fig. 3 illustrates that soluble VCAM-1/Fc complex binding to Jurkat cells did not induce the expression of the mAb 24 epitope. Controls included untreated Jurkat cells (negative control) and cells treated with 5 mmol/L Mg^{2+} and 1 mmol/L EGTA (positive control). PMA treatment, which stimulates leukocyte adhesion to ICAM-1 independent of changes in integrin affinity (15), did not increase mAb 24 binding. Treatment with soluble VCAM-1/Fc or IgG complexes did not alter binding of TS1/22, which recognizes all $\alpha_L\beta_2$ integrins. These data imply that increased $\alpha_L\beta_2$ integrin affinity could not account for the increased strength of Jurkat cell adhesion to the combination of VCAM-1/Fc and ICAM-1/Fc or to ICAM-1/Fc following cross-linking of α_4 integrins.

Cross-linking of α_4 integrins induces clustering of $\alpha_L\beta_2$

After demonstrating that cross-linking α_4 integrins did not alter β_2 integrin affinity, Jurkat cells were treated with soluble VCAM-1/Fc

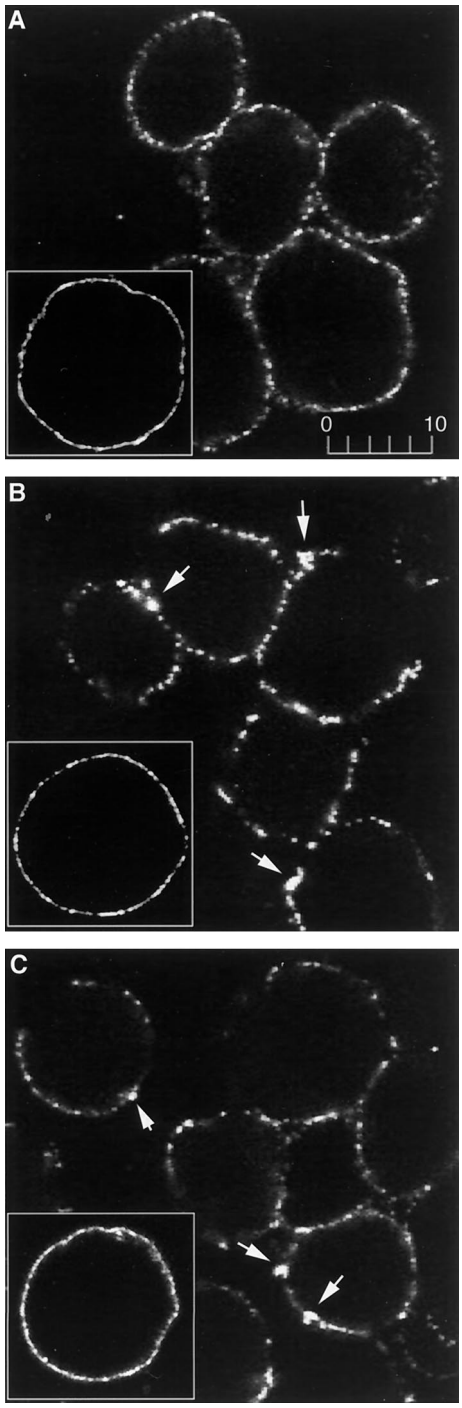

FIGURE 4. Cross-linking $\alpha_4\beta_1$ integrins induces clustering of β_2 integrins on Jurkat cells. Confocal microscopy analysis of $\alpha_L\beta_2$ integrins on untreated (*A*), soluble VCAM-1/Fc-anti-human IgG-treated (*B*), or PMA-treated (*C*) Jurkat cells. Jurkat cells were allowed to adhere to poly-L-lysine-coated dishes for 15 min at 37°C, stained with Ab TS 1/22 on ice for 60 min, and fixed with 0.5% paraformaldehyde. Cy3-conjugated donkey anti-mouse IgG was used as secondary Ab. Mean fluorescence intensity of TS 1/22 was not different in untreated, soluble VCAM and anti-human IgG-treated, and PMA-treated cells (mean fluorescence intensity ± SD 91.7 ± 17.4, 90.3 ± 16.4, and 72.4 ± 23.2, respectively, $n = 5$, p = NS). As a control, distribution of class I MHC was assessed (*inset*) and was not different among treatment groups. Arrows indicate regions of high-intensity $\alpha_L\beta_2$ integrin signal. Scale bar represents 10 μm.

and cross-linking anti-human IgG, or PMA as described above, and the distribution of α_L integrins was visualized by confocal microscopy using mAb TS1/22 and Cy3-conjugated donkey anti-mouse IgG. In untreated cells, α_L integrin staining was distributed more uniformly around the periphery of cells than cells treated with soluble VCAM-1/Fc or PMA (Fig. 4). These treatments resulted in more punctate high intensity fluorescent signals indicative of clustered α_L integrins. Because treatment with soluble VCAM-1/Fc complexes or PMA could not increase the surface expression of $\alpha_L\beta_2$ integrins during the time course of this assay, the difference in the staining pattern between untreated and treated cells was likely due to redistribution of the Cy3 signal induced by clustering of $\alpha_L\beta_2$ integrins, as has been suggested by others (30). Our positive control using PMA was consistent with previous reports (31). As a further control, we also examined the distribution of class I MHC in soluble VCAM-1/Fc-treated and untreated cells. In untreated cells, class I MHC distribution was more uniform than $\alpha_L\beta_2$ integrin. Furthermore, the distribution of class I MHC in the cell membrane was not altered by treatment with soluble VCAM-1/Fc. Clustering of $\alpha_L\beta_2$ integrins may increase their ligand binding strength and thus provide a mechanism by which ligation of $\alpha_4\beta_1$ integrins increases β_2 integrin-mediated adhesion to ICAM-1.

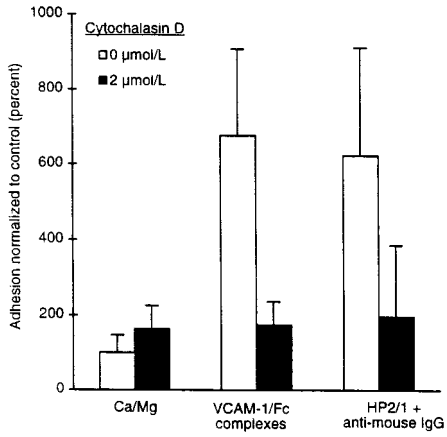

FIGURE 5. Activation of $\alpha_L\beta_2$ integrin adhesion by α_4 integrin signaling is inhibited by cytochalasin D. Jurkat cells in HBSS buffer containing 1 mmol/L of Ca^{2+} and Mg^{2+} were preincubated with buffer alone (Ca/Mg), HP2/1 + anti-mouse IgG, or soluble VCAM-1/Fc complexes in the presence or absence of cytochalasin D (2 μmol/L). Cells were introduced into flow chambers with an ICAM-1/Fc-coated surface (1100 molecules/μm^2), allowed to adhere for 10 min, then graded fluid shear stress was applied. The number of cells remaining adherent after 10 dynes/cm^2 was determined. Data are expressed as a percentage of control adhesion (Ca^{2+}/Mg^{2+}, without cytochalasin D). The same trends were observed at other levels of shear stress.

The ability of integrins to cluster is regulated by the cytoskeleton (31). Cytochalasin D, a disrupter of the actin cytoskeleton (32), was used in detachment assays to determine whether $\alpha_L\beta_2$ integrin adhesion to ICAM-1 induced by cross-linking of α_4 integrins involved cytoskeletal rearrangement. Cytochalasin D abrogated the increase in Jurkat cell adhesion to ICAM-1 that was induced by either soluble VCAM-1/Fc complexes or HP2/1 plus anti-mouse IgG (Fig. 5). Cytochalasin D had minimal effects on basal adhesion.

Human peripheral blood T lymphocytes

This series of experiments was performed to determine whether binding of VCAM-1 by $\alpha_4\beta_1$ integrins can activate β_2 integrins on human peripheral blood T lymphocytes. Initial detachment assays revealed that these leukocytes did not adhere significantly to either VCAM-1/Fc, ICAM-1/Fc, or a combination of both (data not shown). However, when the affinity of T cell $\alpha_4\beta_1$ integrins was

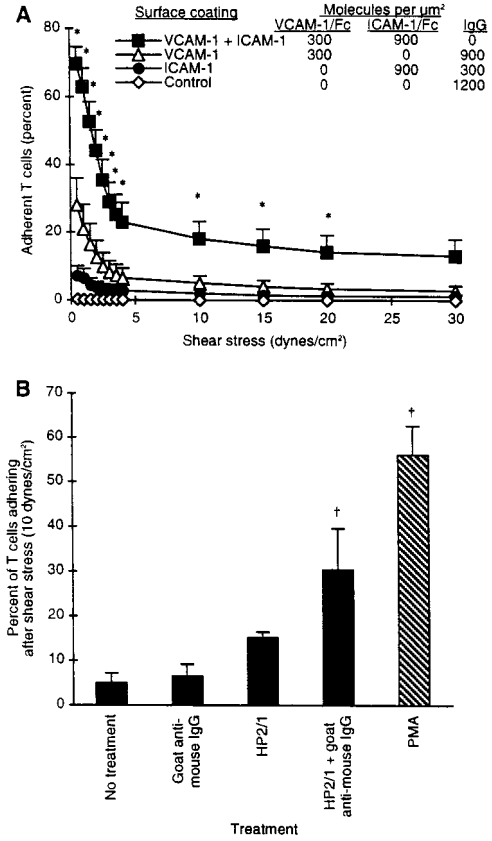

FIGURE 6. Synergistic adhesion of T cells to coimmobilized VCAM-1 and ICAM-1. *A*, Purified human peripheral blood T cells suspended in HBSS buffer containing 1 mmol/L of Ca^{2+}, Mg^{2+}, and Mn^{2+} were allowed to adhere to VCAM-1 + ICAM-1, VCAM-1, ICAM-1, or nonimmune IgG. After 10 min of static adhesion, fluid shear stress was introduced in a stepwise fashion from 0 to 30 dynes/cm^2. The percentage of cells remaining adherent at the end of each shear level is shown. Mean ± SEM of 7–13 samples collected over four experimental days, using T cells from three different subjects. ∗, Significant difference from VCAM-1, determined by ANOVA and Bonferroni-Dunn posthoc test ($p < 0.01$). *B*, α_4 integrins on 1 mmol/L Mn^{2+}-treated human T cells were cross-linked with HP2/1 and goat anti-mouse IgG. The percentage of cells remaining adherent after 10 dynes/cm^2 shear stress was calculated. Mean ± SEM of three experiments. †, Significant difference from untreated cells, determined by ANOVA and Fisher's PLSD posthoc test ($p < 0.05$).

increased by treatment with 1 mmol/L $MnCl_2$, these cells adhered preferentially to VCAM-1/Fc and more strongly to a combination of VCAM-1/Fc and ICAM-1/Fc than to either alone (Fig. 6*A*). The strengthening of T lymphocyte adhesion was synergistic and similar to that seen in Jurkat cells (Fig. 2*A*). To provide further evidence that ligation of α_4 integrins could activate $\alpha_L\beta_2$ integrins, we cross-linked α_4 integrins with HP2/1 and goat anti-mouse IgG. This treatment did not induce adhesion to immobilized ICAM-1/Fc using standard assay buffer (data not shown). However, cross-linking of α_4 integrins on $MnCl_2$-treated human T cells induced adhesion to ICAM-1/Fc (Fig. 6*B*).

Synergistic adhesion of Jurkat cells to coimmobilized FN40 and ICAM-1

To determine whether signals transduced through $\alpha_4\beta_1$ integrins to the $\alpha_L\beta_2$ integrins were ligand specific, we performed detachment assays with FN40. The CS-1 region of fibronectin is an alternative ligand for $\alpha_4\beta_1$ (33) and is contained within the 40-kDa fragment of fibronectin used in our study. Furthermore, this fragment does

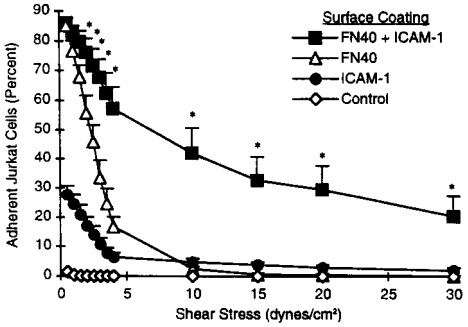

FIGURE 7. Synergistic adhesion of Jurkat cells to coimmobilized FN40 and ICAM-1. Jurkat cells suspended in HBSS buffer containing 1 mmol/L of Ca^{2+}, Mg^{2+} were allowed to adhere to FN40 + ICAM-1, FN40, ICAM-1, or nonimmune IgG. After 10 min of static adhesion, fluid shear stress was introduced in a stepwise fashion from 0 to 30 dynes/cm^2. The percentage of cells remaining adherent at the end of each shear level is shown. Mean ± SEM of seven samples collected over three experimental days. ∗, Significant difference between FN40 + ICAM-1 and FN40, determined by ANOVA and Bonferroni-Dunn posthoc test ($p < 0.01$).

not contain binding sites (RGD) for $\alpha_5\beta_1$ integrin, a fibronectin receptor also present on Jurkat cells. A concentration of FN40 resulting in a strength of adhesion that was qualitatively similar to 100 molecules/μm^2 VCAM-1/Fc was selected and coimmobilized with 1100 molecules/μm^2 ICAM-1/Fc in detachment experiments. Jurkat cells adhered with greater strength to the combination of FN40 and ICAM-1/Fc (Fig. 7) than to either FN40 or ICAM-1/Fc alone.

Discussion

Lymphocyte emigration involves a complex cascade of molecular interactions that must be tightly regulated for a cell to tether, roll, arrest, adhere firmly, and change shape during transendothelial migration (6, 34). In the present paper, we demonstrated strengthening of β_2 integrin-mediated adhesion to ICAM-1 following binding of ligands to high-affinity α_4 integrins. Adhesion of Jurkat cells and Mn^{2+}-stimulated peripheral blood T cells to coimmobilized VCAM-1 and ICAM-1 was stronger than adhesion to either molecule alone. Expression of both VCAM-1 and ICAM-1 is often induced on endothelium at inflammatory sites in chronic diseases. In these situations, both of these adhesion molecules may support firm adhesion of leukocytes. Our observations suggest that expression of both VCAM-1 and ICAM-1 at an inflammatory site provides greater strength of adhesion and greater resistance to detachment of leukocytes than either molecule alone. Cross-talk between α_4 and β_2 integrins may be significant because these molecules could function in parallel during interactions of lymphocytes and monocytes with activated endothelium.

To determine the mechanism by which adhesion was strengthened on coimmobilized VCAM-1 and ICAM-1, we performed assays in which leukocyte α_4 integrins were ligated in solution and allowed to adhere to surface-immobilized ICAM-1. Treatment with soluble VCAM-1/Fc complexes or HP2/1 and a cross-linking secondary Ab induced adhesion of Jurkat cells or human peripheral blood T cells to ICAM-1/Fc. This implies activation of a signaling pathway between α_4 and β_2 integrins upon ligand binding to $\alpha_4\beta_1$ integrins. Cross-talk between other integrins has been previously reported. For example, ligation of transfected $\alpha_v\beta_3$ integrins on K562 cells inhibited the phagocytic function of $\alpha_5\beta_1$ integrins (35), and ligation of transfected $\alpha_{IIb}\beta_3$ integrins on Chinese hamster ovary cells inhibited both $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins (36). In leukocytes, integrin cross-talk has been described as an important

mechanism during the transition from a firmly adherent to a more migratory phenotype. Imhof et al. demonstrated that the velocity of $\alpha_4\beta_1$ integrin-mediated migration across VCAM-1 could be enhanced by the occupancy of $\alpha_v\beta_3$ integrins with PECAM-1 or vitronectin (37). Binding of T lymphocytes to ICAM-1 decreased $\alpha_4\beta_1$ integrin adhesion to fibronectin and enhanced $\alpha_5\beta_1$ integrin-mediated transmigration (22). These data suggest that integrin cross-talk is important in regulating leukocyte transendothelial migration. Our data, on the other hand, suggests that integrin cross-talk may be relevant to an earlier stage in the emigration process and provide an alternative mechanism for regulating firm adhesion to endothelial ligands such as ICAM-1.

Recently, members of the selectin family have been shown to regulate integrin-mediated adhesion. In neutrophils, ligation of L-selectin or P-selectin glycoprotein ligand-1 induces β_2 integrin-mediated cell adhesion to ICAM-1 (23, 38). Similarly, L-selectin ligation on T lymphocytes activates both β_1 and β_2 integrin-mediated cell adhesion to fibronectin and ICAM-1, respectively (11, 12). Like the selectins, $\alpha_4\beta_1$ integrins can mediate rolling interactions with VCAM-1 (7, 8). Thus, while mediating rolling interactions, selectins and/or $\alpha_4\beta_1$ integrins may provide activation signals required to initiate firm adhesion by the β_2 integrins.

In our study, ligation of $\alpha_4\beta_1$ integrins by either soluble VCAM-1/Fc complexes or mAb to the α_4 subunit strengthened cell adhesion to ICAM-1/Fc. The dominant β_2 integrin on Jurkat cells is $\alpha_L\beta_2$. $\alpha_M\beta_2$ integrins were not detected by flow cytometry (data not shown). Cell surface expression of $\alpha_L\beta_2$ integrins on Jurkat cells could not be altered during the time course of our assay. Increased cell-surface expression of an integrin is not required for strengthened adhesion. Rather, integrin-mediated adhesion requires activation of inside-out intracellular signal transduction cascades. Adhesion of integrins to their ligands can be regulated at the level of integrin affinity, whereby activation of the integrin results in a conformational change that stabilizes ligand binding by each integrin heterodimer. Monoclonal Abs that recognize these conformational changes serve as reporters of high-affinity integrins (9). We did not detect expression of the high-affinity β_2 integrin epitope recognized by mAb 24 following stimulation with soluble VCAM-1/Fc complexes, suggesting that the observed increase in adhesion to ICAM-1/Fc was not due to changes in β_2 integrin affinity.

Integrin-mediated adhesion can be regulated independently of affinity modulation. Increases in avidity can strengthen leukocyte adhesion through mechanisms that include clustering of integrins in the plasma membrane. Avidity changes are thought to be regulated by the cytoskeleton. Integrins can be linked to the cytoskeleton via proteins such as α-actinin, filamin, and talin (39). A proposed model of avidity changes involves integrin release from the cytoskeleton to promote diffusion in the plasma membrane and formation of integrin clusters. Once bound to ligand, reestablishment of cytoskeletal connections confers strengthened adhesion (31, 40). In our study, treatment of Jurkat cells with soluble VCAM-1/Fc and anti-human IgG induced clustering of $\alpha_L\beta_2$ integrins. Furthermore, cytochalasin D abolished the activation of β_2 integrin adhesion to ICAM-1 induced by ligation of $\alpha_4\beta_1$ integrins. The effect of cytochalasin D may be due to prevention of the reassociation of β_2 integrins with the cytoskeleton. On the other hand, cytochalasin D may act by preventing formation of the intracellular signaling complexes required for inside-out regulation of β_2 integrin activity.

Recent studies have indicated that ligand specific changes in $\alpha_4\beta_1$ integrin conformation are induced upon ligand binding (41). Furthermore, it is apparent that there are mechanistic differences between $\alpha_4\beta_1$ integrin-mediated adhesion to the CS-1 region of fibronectin and to VCAM-1 (42). These reports suggest that alternative ligands for $\alpha_4\beta_1$ may transduce differential signals into the leukocyte. In our study, coimmobilization of either VCAM-1/Fc or the CS-1-containing FN40 fragment of fibronectin with ICAM-1/Fc resulted in a synergistic increase in strength of adhesion. Therefore, cross-talk between $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins does not appear to depend on the ligand for $\alpha_4\beta_1$. Coupled with our observation that cross-linking of α_4 integrins with mAb HP2/1 can strengthen $\alpha_L\beta_2$-mediated adhesion to ICAM-1, our data suggest that clustering of $\alpha_4\beta_1$ integrins may be sufficient to generate signals to activate the $\alpha_L\beta_2$ integrins.

Signaling mechanisms by which $\alpha_4\beta_1$ integrins communicate with β_2 integrins are likely complex. Our data suggests that high-affinity $\alpha_4\beta_1$ integrins mediate an outside-in signal resulting in the regulation of β_2 integrin adhesive function. Low-affinity $\alpha_4\beta_1$ integrins do not appear to signal because cross-linking of α_4 integrins on peripheral blood T cells could not induce cell adhesion to ICAM-1/Fc in the absence of Mn^{2+}. However, the intracellular signaling pathways activated by ligation of high-affinity $\alpha_4\beta_1$ integrins important for the inside-out regulation of β_2 integrin activity remain unclear. A candidate mediator may be cytohesin-1, a 47-kDa cytoplasmic protein that binds to the β_2 integrin cytoplasmic tail (43). Phosphatidylinositol 3-kinase activation recruits cytohesin-1 to the plasma membrane, where it binds to the β_2 integrin cytoplasmic tail and induces adhesion to ICAM-1 (44). In Jurkat cells, cytohesin-1 coprecipitates with $\alpha_L\beta_2$ but not $\alpha_4\beta_1$ integrins and thus may have a role in inside-out signaling to the β_2 integrin.

In the classical model of leukocyte adhesion and transendothelial migration, integrins are activated during leukocyte tethering and rolling interactions with endothelium (6). The activation of integrin adhesive function is crucial to develop firm or stable adhesion as lymphocytes recirculate or accumulate at sites of inflammation. This activation may be stimulated by endothelial-derived chemokines binding to their G protein-coupled receptors on the leukocyte surface (45, 46). Several recent studies also provide evidence for activation of leukocyte integrins by selectins during selectin-mediated rolling interactions (11, 12). We have demonstrated that VCAM-1 binding to $\alpha_4\beta_1$ integrins can strengthen β_2 integrin-mediated adhesion to ICAM-1. There is considerable evidence for sequential regulation of integrin function during the transition of an adherent cell to a migratory phenotype. Chemokines have been demonstrated to simultaneously cause rapid but transient activation of α_4 integrins and delayed but prolonged activation of β_2 integrins on eosinophils (13). Adhesion of T cells via β_2 integrins decreases $\alpha_4\beta_1$ integrin-mediated adhesion, thereby enhancing $\alpha_5\beta_1$ integrin-mediated transmigration (22). Our novel observation demonstrates a potential mechanism whereby leukocyte integrins may regulate the transition from rolling to firm adhesion during emigration and therefore adds further complexity and combinatorial diversity to the process of leukocyte adhesion and transendothelial migration during inflammation.

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