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

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Abstract. A regulated order of adhesion events directs leukocytes from the vascular compartment into injured tissues in response to inflammatory stimuli. We show that on human T cells, the interaction of the $\beta_2$ integrin leucocyte function–associated antigen-1 (LFA-1) with its ligand intercellular adhesion molecule-1 (ICAM-1) will decrease adhesion mediated by $\alpha$4$\beta$1, and, to a lesser extent, $\alpha$5$\beta$1. Similar inhibition is also seen when T cells are exposed to mAb 24, which stabilizes LFA-1 in an active state after triggering integrin function through divalent cation Mg$^{2+}$, PdBu, or T cell receptor/CD3 complex (TCR/CD3) cross-linking. Such cross talk decreases $\alpha$4$\beta$1 integrin–mediated binding of T cells to fibronectin and vascular cell adhesion molecule-1 (VCAM-1). In contrast, ligand occupancy or prolonged activation of $\beta_1$ integrin has no effect on LFA-1 adhesion to ICAM-1. We also show that T cell migration across fibronectin, unlike adhesion, is mediated solely by $\alpha$5$\beta$1, and is increased when the $\alpha$4$\beta$1-mediated component of fibronectin adhesion is decreased either by cross talk or the use of $\alpha$4-blocking mAb. The ability of mAb 24 Fab’ fragments to induce cross talk without cross-linking LFA-1 suggests signal transduction through the active integrin. These data provide the first direct evidence for cross talk between LFA-1 and $\beta_1$ integrins on T cells. Together, these findings imply that activation of LFA-1 on the extravasating T cell will decrease the binding to VCAM-1 while enhancing the subsequent migration on fibronectin. This sequence of events provides a further level of complexity to the coordination of T cell integrins, whose sequential but overlapping roles are essential for transmigration.

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with little or no contribution from LFA-1 becomes activated, adhesion is mediated through LFA-1 (ICAM-1) or by activation mAbs. Such sustained activation of LFA-1 is shown to downregulate the adhesion (ICAM-1) or by activation mAbs. Therefore, we have provided direct evidence of cross talk on T cells between LFA-1 and another integrin. The avid state of the β2 integrin, LFA-1, can be maintained by the ligand intercellular adhesion molecule-1 (ICAM-1) or by activation mAbs. Such sustained activation of LFA-1 is shown to downregulate the adhesion through α4β1 and, to a lesser extent, through α5β1 to the ligands fibronectin and vascular cell adhesion molecule-1 (VCAM-1) (α4β1 alone). The result, phenotypically, is a less adhesive, more migratory T cell. Therefore, we have demonstrated another way in which integrin activities may be regulated.

Materials and Methods

Preparation of T Lymphoblasts

Peripheral blood mononuclear cells were prepared from single donor leukocyte buffy coats by centrifugation through Lymphoprep® (Pharmacia Diagnostics AB, Uppsala, Sweden). T cells were expanded from this population, containing 65% CD8 and 35% CD4, by culturing in RPMI 1640 plus 10% FCS (GIBCO BRL, Paisley, UK) in the presence of phytohaemagglutinin (Murex Diagnostics, Dartford, UK) at 1 μg/ml for 72 h as previously described (Dransfield et al., 1992a). Cells were then washed and maintained for 1–2 wk in medium supplemented with 20 ng/ml recombinant IL-2 (Euro Cetus UK Ltd., Harefield, UK). The cells, which were used between days 10 and 14, were a 99% CD3 population, containing 65% CD8 and 35% CD4 cells. The population was negative for the natural killer cell marker CD56.

mAbs and Other Reagents

mAb 9252 (CD11a; LFA-1α subunit, nonblocking) was purchased from Becton Dickinson (Oxford, UK). mAbs 38 (CD11a; LFA-1 α subunit function-blocking), and 24 (CD11/CD18; β2 integrin activation reporter) (Dransfield et al., 1992b; Dransfield and Hogg, 1989) and 52U (control antibody) were prepared in this laboratory, and purified from ascites or tissue culture supernatant by protein A-Sepharose chromatography (Ey et al., 1978). mAbs HPI/2 (CD49d; α4 subunit function-blocking) and TS2/16 (CD29; β1 subunit-activating) were gifts from R. Lobb (Biogen, Inc., Cambridge, MA). mAb SAM-1 (CD49e; α5 subunit-blocking) was purchased from Eurogenetics (Hampton, UK). mAb 7.2 (CD49d; α4 subunit, nonblocking) was gifted from J. Marshall (Imperial Cancer Research Fund, London, UK). mAb UCHT2 (CD5) was a gift from P. Beverley (University College, London, UK). mAb G19.4 was a gift from Bristol Myers-Squibb Pharmaceuticals (Princeton, NJ). Rabbit anti–mouse IgG was purchased from Sigma Chemical Co. (Poole, UK).

ICAM-1Fc was produced as a chimeric protein, consisting of the five extracellular domains of ICAM-1 fused to the Fc fragment of human IgG1 (Berendt et al., 1992). VCAM-1Fc, produced as a chimera consisting of the two amino-terminal domains of human VCAM-1 fused to the Fc fragment of human IgG1 (Jakubowski et al., 1995), was a gift from R. Lobb. Fibronectin (0.1% solution from human plasma) was purchased from Sigma Chemical Co., and cytochalasin D from GIBCO BRL. The fluorescent cell label 2',7'-bis-[carboxyethyl]-5(6)-carboxyfluorescein (BCECF-AM) was purchased from Calbiochem Corp. (Nottingham, UK). All other chemicals and reagents were purchased from Sigma Chemical Co.

Ligand-coated Beads

A modified protocol (Pyszniak et al., 1994) was developed in which 200 μl (10³) of 3.2-μm polystyrene beads (Sigma Chemical Co.) were washed twice in distilled water, followed by two further washes and resuspension in 0.1 M bicarbonate buffer, pH 9. Fibronectin, ICAM-1, VCAM-1, or BSA as control were added to these beads to a final concentration of 10 μg/ml. The beads were rotated at room temperature once in PBS, and blocked with 0.1% denatured BSA for 2 h at room temperature while being rotated. The beads were then washed twice in 20 mM Hepes, 140 mM NaCl, 2 mg/ml glucose, pH 7.4 (assay buffer), containing 3 mM Mg²⁺/2 mM EGTA, for use in the adhesion experiments as described.

Cell Bead Attachment Assay

Multwell Lab-Tek® Chamber Slides (Nunc, Inc., Naperville, IL) were left uncoated as controls or coated with either ICAM-1Fc (10 μg/ml in PBS) or rabbit anti–mouse Ig (1:100 dilution in PBS) overnight at 4°C. The next day, mAb tissue culture supernatant was added to wells precoated with anti-mouse Ig and left on ice for 1 h. Wells were washed twice with PBS, and nonspecific binding sites were blocked with 0.1% denatured BSA for 2 h at room temperature. Cells (150 μl of 2 x 10⁶/ml) in assay buffer (see above) containing 3 mM Mg²⁺/2 mM EGTA were added to the wells and allowed to settle on ice for 15 min. Freshly prepared ligand-coated beads (see above) were added to the wells at 1:1 bead-to-cell ratio in 50 μl. After 30 min at 37°C, the unbound beads and cells were removed with four washes in warmed assay buffer. Bound cells were fixed with 1% formaldehyde in PBS for 20 min at room temperature. Cells were then stained with haematoxylin for 10 min. Beads and cells were counted per high power field (×40 oil immersion objective; Carl Zeiss, Inc., Thornwood, NY), and the number of beads per 100 cells was determined (attachment index).

Cell Attachment Assays

Flat-bottomed Immulon-1® 96-well plates (Dynatech Labs., Inc., Chantilly, VA) were precoated with 50 μl fibronectin (20 μg/ml), VCAM-1Fc (7 μg/ml), or ICAM-1Fc (2.4 μg/ml) in PBS overnight at 4°C. The plates were blocked with 2.5% BSA in PBS for 2 h at room temperature and then washed four times in assay buffer (see above) at 4°C. T cells were washed three times in assay buffer and labeled with 2.5 μM BCECF-AM in the same buffer for 30 min at 37°C, followed by two further washes. T cells (2 x 10⁵ cells) were treated with 3 mM Mg²⁺/2 mM EGTA, 50 mM phorbol-12,13-dibutyrate (PdBu), or CD3 mAb at indicated levels, as well as inhibitors and mAbs in 100 μl assay buffer. Ca²⁺ and Mg²⁺ were included at 0.4 mM for experiments involving PdBu or T cell receptor/CD3 complex (TCR/CD3) cross-linking with mAb G19.4. Blocking mAbs were titrated on T cells by FACS® analysis (Becton Dickinson, Mountain View, CA) and used at saturating concentrations to block T cell function for fibronectin- and VCAM-1-binding assays, all wells containing anti-LFA-1 mAb 38 at function-blocking concentrations of 10 μg/ml. This prevents cells aggregating via LFA-1/ICAM-1 interactions, which would cause spurious high binding to β1 ligand through the piggy-back interaction of nonadherent cells with truly adherent cells. Plates were incubated for 15 min on ice, followed by centrifugation at 40 g for 1 min, before 40-min incubation at 37°C. Nonadherent cells were removed by washing four times in warmed assay buffer (150 μl/well). Adhesion was quantified by recording emission at 530 nm, after excitation at 485 nm, using a Fluoroskan® II (Labsystems, Inc., Basingstoke, UK), and by expressing the reading for each well as a percentage of the total emission before incubation.

Transmigration Assays

Assays were performed in 20 mM Hepes, 140 mM NaCl, 2 mg/ml glucose, pH 7.4, 0.05% BSA, 3 mM Mg²⁺/2 mM EGTA (transmigration buffer) using 6.5-mm-diam Transwell® plates (Costar Corp., Cambridge, MA). The upper and lower surfaces of the inserts were coated with fibronectin at concentrations ranging from 0 to 50 μg/ml in PBS overnight at 4°C. The inserts were positioned in wells containing 600 μl transmigration buffer. Cells were then plated in the insert at a concentration of 5 x 10⁶ cells in 100 μl transmigration buffer with appropriate mAbs. The mAbs were used at the following final concentrations: mAb HPI/2 at 0.7 μg/ml, mAb 7.2 at 5 μg/ml, mAb SAM-1 at 5 μg/ml, mAb 24 at 5 μg/ml, and mAb 52U at 5 μg/ml. The anti–LFA-1 mAb 38 was added to all inserts at function-blocking concentrations of 10 μg/ml to prevent a spurious decrease in migration due to cell aggregation when LFA-1 is activated. The plates were then incubated for 6 h at 37°C. The bottom surface of the insert was then
scraped to release migrated but adherent cells into the bottom well, and the migrated cells were counted in a hemocytometer. Nine grids (0.1 mm² per grid) were counted per well, and readings were averaged from duplicate samples. All assays were performed in duplicate, and each experiment was repeated a minimum of four times.

Results

In this study, we have investigated cross influences on function between the β1 integrins and LFA-1 on T cells. As one method of activating these leukocyte integrins, we treated T cells with 3 mM Mg²⁺/2 mM EGTA (Dransfield et al., 1992a). For the β2 integrin, LFA-1, the advantage of such treatment is that it directly alters the integrin ectodomain, bypassing the requirement for an intracellular stimulus (Stewart et al., 1996). This form of LFA-1 is considered to be of high affinity because it is able to bind soluble ICAM-1 (Stewart et al., 1996). There have been both positive and negative reports of the ability of Mg²⁺/EGTA to induce fibronectin receptor–mediated adhesion (Shimizu and Mobley, 1993; Luque et al., 1996). In this study, we show that T cells do bind fibronectin, immobilized either on plates or on beads, in an Mg²⁺-dependent manner. To examine further the generality of cross-influences between these integrins, we also investigated T cells stimulated with phorbol ester or by TCR/CD3 cross-linking. Both of these stimuli act from within the cell to activate integrins, so called inside-out signaling, and may be considered more representative of the in vivo situation.

Adhesion of T Cells to ICAM-1 Will Decrease Binding of Fibronectin-coated Beads

To determine the effect of LFA-1 ligation on the function of the β1 integrins on the same T cell, we developed a ligand-coated bead–binding system. T cells were adhered to immobilized ICAM-1 via LFA-1, or to a control substrate, and their ability to bind beads coated with ligand for the β1 integrins, α4β1 and α5β1, was then investigated. Fibronectin-coated beads were bound by T cells adherent to the control substrate, anti-CD5 mAb, immobilized on plastic (Fig. 1 A), and the specificity of adhesion was demonstrated by blocking bead-binding with a combination of α4 and α5 function–blocking mAbs (Fig. 1 B). When binding of the fibronectin-coated beads was quantified, there was a decreased level of fibronectin bead-binding when T cells were adherent to ICAM-1 (Fig. 2 A) (inhibition: 65.0 ± 23.4% = mean ± SD; n = 6). This result demonstrated that, on human T cells, the interaction of LFA-1 with its ligand ICAM-1 could downregulate the function of the β1 integrins. In contrast, T cells adhered to anti–LFA-1 mAb bound beads at a similar level as T cells adherent to control mAb. This indicated that the LFA-1 inhibitory effect could not be mimicked by cross-linking LFA-1 with immobilized CD11a mAb 38 (Fig. 2 A). Conversely, there was no difference between the ability of T cells adherent to anti-CD5 mAb, fibronectin, or ICAM-1 to bind ICAM-1–coated beads (Fig. 2 B), indicating that adhesion to immobilized fibronectin did not alter the extent of ICAM-1 bead binding by LFA-1. This is the first evidence that LFA-1 could dominate the activity of the fibronectin-binding receptors and that the reverse situation did not hold.

Adhesion of T Cells to ICAM-1 Decreases Binding of Fibronectin- and VCAM-1–coated Beads by Downregulating α4β1 Activity

We then looked at the effects of LFA-1 ligand–binding on each of the T cell fibronectin receptors, α4β1 and α5β1. Both of these integrins can be involved in T cell binding to fibronectin, and, if one is blocked, the other can partially
for fibronectin-coated beads is lower, and is reduced only when T cells are adherent to ICAM-1, the binding index through a mixture of blocking mAbs, showing that T cells bind these beads blocking mAb, or completely with a combination of both blocking mAbs, demonstrating that the binding activity of α4β1 has been compromised. The binding by T cells of VCAM-1–coated beads, which is mediated exclusively by α4β1, reveals a similar downregulation when T cells are adherent to ICAM-1 as compared to anti–LFA-1 mAb (Fig. 4).

**Activation of the β2 Integrin LFA-1 on T Cells Inhibits Their β1-Mediated Binding to Fibronectin**

The inhibitory effect of LFA-1 on β1-mediated ligand-binding could not be demonstrated by cross-linking LFA-1 with an anti–LFA-1 mAb, but required binding to ligand ICAM-1. This suggested that high affinity LFA-1 rather than receptor cross-linking was necessary for cross talk. This led to the development of an assay in which T cells were first stimulated with Mg2+/EGTA, TCR/CD3 cross-linking, or PdBu and then exposed to mAb 24, which holds LFA-1 in an active conformation as if occupied by ligand (Dransfield et al., 1992). mAb 24 caused increased T cell binding to ICAM-1 after titration of Mg2+ (Fig. 5 A), CD3 mAb G19.4 (Fig. 5 B), and PdBu (not shown). In contrast, mAb 24 caused inhibition of T cell binding to fibronectin after TCR/CD3 cross-linking (Fig. 6 A) or Mg2+/EGTA (data not shown). Monovalent Fab' fragments of mAb 24 produced the same degree of inhibition as bivalent mAb 24 (data not shown). This demonstrated that activation or ligand occupancy of LFA-1 in the absence of clustering is sufficient to alter fibronectin-mediated adhesion. mAb KIM185 (CD18; β2-activating) behaved similarly to mAb 24, depressing binding of T cells to fibronectin while enhancing adhesion to ICAM-1 (data not shown).

**The Effect of LFA-1 Activation on T Cells Is Mediated Predominantly through α4β1**

Because T cells bind to fibronectin through both α4β1 and α5β1, we analyzed the effects of LFA-1 activation individually on these integrins using function-blocking mAbs and either TCR/CD3 cross-linking (Fig. 6, B and C) or Mg2+/EGTA (data not shown) to stimulate adhesion. Prolonged activation of LFA-1 with mAb 24 had only a small effect on total fibronectin-binding (Fig. 6 A) and on α5β1-mediated adhesion (Fig. 6 B), but had a much greater effect on α4β1-mediated adhesion (Fig. 6 C). Similar levels of α4β1 inhibition by mAb 24 were seen when the integrins were activated with PdBu or Mg2+/EGTA (Fig. 7). In addition, there was no effect of the nonfunction-altering anti–LFA-1 mAb G25.5, which again emphasized the requirement for LFA-1 activation (Fig. 7). Under equivalent activating conditions, mAb 24 and the β2 integrin–activating mAb KIM185 decreased α4β1-mediated adhesion to VCAM-1 to the same extent as to fibronectin (data not shown). Together, these results reinforced the findings that α4β1 function is particularly sensitive to the state of LFA-1 activation.

**Activation of β1 Integrins on T Cells Has No Effect on LFA-1 Binding to ICAM-1**

We then reversed the situation to investigate the effect on
β2 integrin–mediated adhesion of maintaining β1 integrins in an active state, using the β1 integrin–stimulating mAb TS2/16. This mAb increased binding to fibronectin after the three activating treatments (Fig. 8 A), but had no effect on β2-mediated binding to ICAM-1 (Fig. 8 B), confirming that the β1 integrins were unable to influence the ligand-binding activity of LFA-1.

**Inhibition of α4β1 with Blocking mAbs or by LFA-1 Activation Increases α5β1-Mediated Migration**

The effects of LFA-1–mediated cross talk on α4β1- and α5β1-mediated T cell migration on fibronectin were then investigated. Using the Transwell® system, we established that T cells undergo random migration on fibronectin us-

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*Figure 3.* Fibronectin bead–binding mediated by α4β1 is differentially inhibited when T cells are adherent to ICAM-1. Cultured human T cells treated with 3 mM Mg²⁺/2 mM EGTA were adhered to plastic coated with anti-CD5 (mAb UCHT2) (A) or ICAM-1 (B) and incubated with fibronectin-coated beads. Bead attachment was assessed in the presence or absence of the α4 and α5 function–blocking mAb HP1/2 and SAM-1 either alone or in combination, as previously. The data are expressed as binding index (beads bound/100 cells), and are the mean of six high-power fields ± SEM. One representative experiment of three is shown.

*Figure 4.* Inhibition of VCAM-1 bead–binding when T cells adhere to ICAM-1. Cultured human T cells treated with 3 mM Mg²⁺/2 mM EGTA were adhered to plastic coated with anti-CD5 (mAb UCHT2), anti-LFA-1 (mAb 38), or ICAM-1 and incubated with VCAM-1–coated beads (hatched bars). Bead attachment was prevented by the α4-blocking mAb HP1/2 (open bars). Data are expressed as binding index (beads bound/100 cells) and represent the mean of six high-power fields ± SEM. One representative experiment of three is shown.
Figure 5. Prolonged activation of LFA-1 by mAb 24 increases T cell adhesion to ICAM-1 induced either by Mg$^{2+}$ and 2 mM EGTA (A) or by TCR/CD3 cross-linking through CD3 mAb G19.4 in the presence of Ca$^{2+}$ and Mg$^{2+}$ at 0.4 mM (B). The LFA-1 activation antibody, mAb 24 (○) or mAb 52U (IgG1 isotype control) (●) were used at 20 μg/ml. Specificity of adhesion was shown by block of ICAM-1 binding with mAb 38 (LFA-1–function blocking, 10 μg/ml) (▲). Data represent means of triplicates ± SD. One representative experiment of three is shown.

Figure 6. Prolonged activation of LFA-1 blocks α4β1- and to a lesser extent α5β1-mediated binding to fibronectin. Adhesion to fibronectin was induced by TCR/CD3 cross-linking through CD3 mAb G19.4 in the presence of Ca$^{2+}$ and Mg$^{2+}$ at 0.4 mM. (A) Adhesion mediated by α4β1 and α5β1 together. (B) α5β1-mediated adhesion after the α4β1 component had been blocked with mAb HP1/2 (0.5 μg/ml). (C) α4β1-mediated adhesion after the α5β1 component had been blocked with mAb SAM-1 (0.5 μg/ml). Adhesion was assessed in the presence of the LFA-1 activation antibody, mAb 24 (open symbols), or the isotype-matched control antibody, mAb 52U (closed symbols), used at 20 μg/ml. Data represent means of triplicates ± SD. One representative experiment of three is shown. The specificity of the adhesion is shown by the block achieved using α4- and α5-blocking mAbs as indicated.
Investigation of the Mechanism of LFA-1 Cross Talk

Treatment of T cell LFA-1 with Mg^{2+}/EGTA directly induces a high affinity form of the integrin that is able to bind soluble ICAM-1 (Stewart et al., 1996). Therefore, we looked at the ability of β1 integrins to adopt a high affinity state. However, treatment with Mg^{2+}/EGTA yielded no detectable binding of soluble fibronectin or VCAM-1, even when these ligands were used at concentrations up to 1.2 mg/ml. In contrast, 0.5 mM Mn^{2+} was able to induce fibronectin (α4β1 and α5β1)– and VCAM-1 (α4β1)–binding to both T cells and Jurkat cells (data not shown), as has been reported by others (Jakubowski et al., 1995; Gomez et al., 1997). Similarly, while 0.5 mM Mn^{2+} was able to induce expression of the β1 activation reporter epitopes recognized by mAb 15/7 (Yednock et al., 1995) or mAb HUTS-21 (Luque et al., 1996), no expression of these epitopes was observed with Mg^{2+}/EGTA treatment (data not shown). These findings imply that the fibronectin-binding integrins are in a low affinity state after all three methods of stimulation, and that LFA-1 cross talk causes inhibition of postreceptor occupancy events, rather than direct modulation of receptor affinity.

We next tested the possibility that LFA-1 activation might be influencing a cytoskeletal event. Although mAb 24 decreased the overall level of α4β1-mediated adhesion to fibronectin, there was no change in the sensitivity of binding to cytochalasin D (Fig. 10). Therefore, LFA-1 cross talk affects an event in cell adhesion after receptor occupancy but before changes in the actin cytoskeleton, and is independent of both.

Discussion

This study was undertaken to examine the functional interaction on T cells between LFA-1 and the β1 integrin fibronectin receptors α4β1 and α5β1. The main findings are that (a) the occupation of T cell LFA-1 by its ligand ICAM-1 decreases the binding of α4β1 to ligands fibronectin and VCAM-1; (b) this inhibitory cross talk also results from the prolonged activation of LFA-1 induced by the activation reporter mAb 24 in combination with several T cell adhesion–inducing protocols; (c) the adhesive activity of α5β1 is affected to a lesser extent; (d) while active LFA-1 downregulates the avidity of α4β1, the reverse does not occur, as neither β1 integrin–activating mAb TS2/16 nor β1-mediated binding to fibronectin affected the avidity of LFA-1; and (e) downregulation of α4β1 activity increases the efficiency of α5β1-mediated migration on fibronectin. Therefore, we have demonstrated differential regulation of two integrin subclasses and a hierarchy of binding with either an activation mAb, the ability of migration was blocked with an LFA-1 activation reporter mAb 24 (Fig. 9). Similarly, while 0.5 mM Mn^{2+} has been reported by others (Jakubowski et al., 1995; Gomez et al., 1997). Similarly, while 0.5 mM Mn^{2+} was able to induce fibronectin (α4β1 and α5β1)– and VCAM-1 (α4β1)–binding to both T cells and Jurkat cells (data not shown), as has been reported by others (Jakubowski et al., 1995; Gomez et al., 1997). Similarly, while 0.5 mM Mn^{2+} was able to induce expression of the β1 activation reporter epitopes recognized by mAb 15/7 (Yednock et al., 1995) or mAb HUTS-21 (Luque et al., 1996), no expression of these epitopes was observed with Mg^{2+}/EGTA treatment (data not shown). These findings imply that the fibronectin-binding integrins are in a low affinity state after all three methods of stimulation, and that LFA-1 cross talk causes inhibition of postreceptor occupancy events, rather than direct modulation of receptor affinity.

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integrin usage in which the β2 integrin LFA-1 will suppress the function of β1 integrins, particularly α4β1.

Previous studies have demonstrated the involvement of α4β1 in leukocyte adhesion to but not migration across endothelium, and of LFA-1 as the chief integrin in transendothelial migration (van Epps et al., 1989; Oppenheimer-Marks et al., 1991; Moser et al., 1992). Furthermore, in vitro experiments during flow have emphasized the requirement that an integrin hierarchy allow coordinated migration of lymphocytes across the endothelium into the tissues (Butcher and Picker, 1996). Our finding that active LFA-1 is able to decrease the ligand-binding activity of α4β1 has direct implications for the sequential activity of these integrins in such an adhesion cascade; LFA-1 may function optimally in the absence of α4β1 adhesion, allowing the T cell to deadhere from the apical surface of the endothelium and transmigrate. Our findings also argue against a redundancy among integrin-ligand pairs in leukocyte transmigration, and imply specific roles for each integrin.

In this study, we have demonstrated that, in contrast to adhesion, the migration of activated T cells on fibronectin is mediated by α5β1 with no contribution from α4β1. In addition, suppressing α4β1 activity on T cells either by mAb 24 or α4 function-blocking mAbs enhanced the level of α5β1 migration, particularly at low fibronectin levels. This may reflect the compensatory increase in α5β1 adhesion, with its migratory potential, when binding through
the nonmigratory α4β1 is blocked. Another possibility is that the enhanced migration by α5β1 is due to removal of a restraint imposed by α4β1. The importance of strength of adhesion in regulating cell migration is well documented (Huttenlocher et al., 1996; Palecek et al., 1997), suggesting that firm adhesion by both α4β1 and α5β1 may make conditions suboptimal for migration. Alternatively, α4β1 may be involved in a more specific inhibition of α5β1 function, as has been described in the control of metalloproteinase expression in fibroblasts (Huhtala et al., 1995). The promotion of migratory behavior by α5β1 through loss of α4β1-binding activity is in keeping with its more prominent role within the tissues after successful negotiation of T cells across the endothelium (Miyake et al., 1992). Therefore, a hierarchy of integrin activity may feature at this later stage of the adhesion cascade, with LFA-1 providing a link between α4β1 and α5β1.

The mechanism for LFA-1 downregulation of α4β1 was explored in several ways. We first established that there was no alteration in expression of either α4β1 or α5β1 during the experimental period (data not shown). Furthermore, confocal microscopy using mAbs specific for α4β1, α5β1, and the β1-activation reporter mAb 15/7 indicated that avid LFA-1 did not cause β1 integrin redistribution on the T cell membrane (data not shown). In addition, although stimulation of T cells with Mg^{2+}/EGTA induces high affinity LFA-1 (Stewart et al., 1996), none of the three stimulating protocols induced high affinity α4β1 or α5β1. This implied that cross talk was not affecting high affinity β1 integrins. Together, these findings suggested...
LFA-1 cross talk was evident after several different adhesion-inducing protocols, showing that the phenomenon was not stimulus specific. The fact that cross talk was dependent on ICAM-1 or mAb 24 indicated that occupancy of LFA-1 was a prerequisite. Although the signaling pathways activated upon engagement of the β2 integrins are not well understood, certain observations suggested that cross talk did activate specific intracellular signaling pathways. Cross talk was not observed using the Jurkata T cell line, which is known to have a defect in LFA-1 signaling (Mobley et al., 1994). In addition, cross talk was induced by mAb 24 Fab' fragments but not by immobilized anti–LFA-1 mAb, emphasizing the requirement for a mechanism beyond LFA-1 clustering. For α5β1 on human fibroblasts, although clustering by mAbs of integrin on beads induced phosphorylation and accumulation of p125 focal adhesion kinase and tensin, ligand occupancy recruited further cytoskeletal proteins to the signaling complex (Miyamoto et al., 1995a,b). One speculation is that the targets of LFA-1 cross talk may be the proteins providing the link between integrins and actin. However, several observations suggested that cross talk does not represent a simple sequestering of such intracellular proteins. First, integrin activity operates in one direction only, so prolonged activation of the β1 integrins using mAb TS2/16 does not alter LFA-1 binding to ICAM-1. Second, LFA-1 predominantly affects the activity of α4β1, despite a sixfold abundance of α4β1 over α5β1 (data not shown). Future work will address the role of potential integrator molecules in the cross talk phenomenon.

In summary, we describe inhibition of α4β1-binding activity in T cells as a consequence of LFA-1 activation. A speculation is that deadhesion of α4β1 from the apical surface of the endothelium is required for LFA-1–mediated migration across endothelium to proceed. Another observation is that T cell migration on fibronectin is mediated by α5β1, and that this migration is enhanced by interfering with α4β1 adhesion. LFA-1 might provide a link between α4β1 and α5β1 by uncoupling the former in order to enhance migration by the latter. While the actual mechanism by which cross talk is achieved is unclear, our findings implicate a downstream signaling event brought about by maintaining LFA-1 in a highly avid state.

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References

Alon, R., P.D. Kassner, M.W. Carr, E.B. Finger, M.E. Hemler, and T.A. Springer. 1995. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J. Cell Biol. 128:1243–1253.

Bargatze, R.F., M.A. Jutla, and E.C. Butcher. 1995. Distinct roles of L-selectin and integrins α4β7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. Immunity. 3:99–108.

Berendt, A.R., A. McDowall, A.G. Craig, P.A. Bates, M.J.E. Sternberg, K. Blystone, et al., 1996; Pacifici et al., 1996), and protein kinase C, implicated in some previous cross talk studies (Blystone et al., 1994; Pacifici et al., 1994), were not involved in this phenomenon (data not shown).

that the β1 integrins had not undergone a detectable alteration in affinity nor been redistributed or shed from the cell surface, and that LFA-1 cross talk was targeting events after ligand-binding. This result is in keeping with other studies in which cross talk is ultimately dependent on the presence of the β subunit cytoplasmic tail and steps subsequent to modulation of integrin affinity (Blystone et al., 1994; Díaz-González et al., 1996).

It seemed possible that the cytoskeleton was a target of LFA-1–mediated cross talk because both α4β1- and α5β1-mediated adhesion were more sensitive to changes in actin than was adhesion through LFA-1 (data not shown). However, for β1 integrin–mediated adhesion, the similarity in cytochalasin D sensitivity of mAb 24–treated and untreated cells and the synergism between suboptimal doses of cytochalasin D and mAb 24 in the inhibition of α4β1-mediated binding to fibronectin (data not shown) supported the evidence that inhibition occurs upstream of cytoskeletal changes. These results implied that cross talk affects an event in cell adhesion occurring after receptor occupancy but before actin-mediated cytoskeletal changes, and independent of both. In addition, protein kinase A, associated with LFA-1 signaling and deadhesion (Rovere et al., 1996), and protein kinase C, implicated in some previous cross talk studies (Blystone et al., 1994; Pacifici et al., 1994), were not involved in this phenomenon (data not shown).

Figure 10. Inhibition of α4β1-mediated adhesion by mAb 24 does not alter the sensitivity of adherent T cells to cytochalasin D. Integrin α5β1 was blocked with SAM-1, as previously, allowing α4β1 adhesion to be investigated in isolation. Adhesion was stimulated with 3 mM MgCl2/2 mM EGTA in the presence of the LFA-1–activation antibody, mAb 24 (○), or the isotype-matched control antibody, mAb 52U (●), as previously. Cytochalasin D was used at 0–10 μg/ml (0–20 μM). Specificity of the adhesion was shown by blocking with mAb HP1/2 (anti-α4) (■). Data represent means of triplicates ± SD. One representative experiment of four is shown.

The Journal of Cell Biology, Volume 138, 1997

1446
