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Dual Role of the Actin Cytoskeleton in Regulating Cell Adhesion Mediated by the Integrin Lymphocyte Function-associated Molecule-1

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Intracellular signals are required to activate the leukocyte-specific adhesion receptor lymphocyte function-associated molecule-1 (LFA-1; CD11a/CD18) to bind its ligand, intracellular adhesion molecule-1 (ICAM-1). In this study, we investigated the role of the cytoskeleton in LFA-1 activation and demonstrate that filamentous actin (F-actin) can both enhance and inhibit LFA-1-mediated adhesion, depending on the distribution of LFA-1 on the cell surface. We observed that LFA-1 is already clustered on the cell surface of interleukin-2/phytohemagglutinin-activated lymphocytes. These cells bind strongly ICAM-1 and disruption of the actin cytoskeleton inhibits adhesion. In contrast to interleukin-2/phytohemagglutinin-activated peripheral blood lymphocytes, resting lymphocytes, which display a homogenous cell surface distribution of LFA-1, respond poorly to intracellular signals to bind ICAM-1, unless the actin cytoskeleton is disrupted. On resting peripheral blood lymphocytes, uncoupling of LFA-1 from the actin cytoskeleton induces clustering of LFA-1 and this, along with induction of a high-affinity form of LFA-1, via "inside-out" signaling, results in enhanced binding to ICAM-1, which is dependent on intact intermediate filaments, microtubules, and metabolic energy. We hypothesize that linkage of LFA-1 to cytoskeletal elements prevents movement of LFA-1 over the cell surface, thus inhibiting clustering and strong ligand binding. Release from these cytoskeletal elements allows lateral movement and activation of LFA-1, resulting in ligand binding and "outside-in" signaling, that subsequently stimulates actin polymerization and stabilizes cell adhesion.

INTRODUCTION

The lymphocyte function-associated molecule-1 (LFA-1)[1] is an adhesion receptor that belongs to the β_2 family of integrins (CD11/CD18). It consists of an α-chain and a β-chain which are noncovalently linked (Kurzinger *et al.*, 1981). LFA-1 is a leukocyte-specific integrin expressed on the cell surface of most leukocytes and coordinates distinct adhesive and signaling interaction in the immune system such as T cell-mediated killing, T helper cell and B cell responses, natural killer cell activity, monocyte-mediated antibody-dependent cytotoxicity, and leukocyte adhesion to endothelial cells (Springer, 1990; Martz, 1987). These highly dynamic interactions are accomplished by interaction of LFA-1 with intercellular adhesion molecule- (ICAM) 1 (Marlin and Springer, 1987), ICAM-2 (Staunton *et al.*, 1989) and ICAM-3 (de Fougerolles and Springer, 1992; Fawcett *et al.*, 1992; Vazeux *et al.*, 1992; de Fougerolles *et al.*, 1993).

LFA-1 expressed by leukocytes is generally not functional and must be activated to bind its ligand. Triggering of the T cell receptor/CD3 complex (TCR/CD3) or stimulation of leukocytes with phorbol 12-myristate 13-acetate (PMA) induces intracellular signals that activate LFA-1 to bind to its ligand (often referred as "inside-out" signaling; Martz, 1987; Dustin and Springer, 1989; van Kooyk *et al.*, 1989; Hynes,

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[1] Abbreviations used: F-actin, filamentous actin, CLSM, confocal laser scanning microscopy.

1992). As shown for PMA, these intracellular signals result in LFA-1 adhesion receptors that have a 200-fold higher affinity for binding of ICAM-1 than of the nonactivated receptors (Lollo *et al.*, 1993). Consistent with this observation, PMA has been shown to increase the diffusion rate of LFA-1, according to the lack of cytoskeletal constraints on LFA-1 (Kucik *et al.*, 1996), suggesting that PMA activation causes a temporary dislodgment of LFA-1 from the cytoskeleton and thereby induces an active conformation (high-affinity state) of LFA-1. We previously demonstrated that LFA-1 can only significantly bind to ICAM-1 when it is expressed in clusters at the cell surface (van Kooyk *et al.*, 1994). Clustering of integrin molecules is thought to enhance the avidity of integrin–ligand interaction (Figdor *et al.*, 1990; van Kooyk *et al.*, 1994). It has recently been suggested for $\alpha_{IIb}\beta_3$ that ligand binding can induce clustering of integrins (Fox *et al.*, 1996). We have demonstrated that a unique antibody NKI-L16, which recognizes a Ca^{2+}-dependent epitope on LFA-1, is only expressed by clustered LFA-1, and can therefore report the distribution of LFA-1 at the cell surface (Keizer *et al.*, 1988; van Kooyk *et al.*, 1994). LFA-1-mediated adhesion to ICAM-1 can thus be regulated both by changes in avidity (clustering) and affinity (active conformation) of LFA-1 (Lollo *et al.*, 1993; van Kooyk *et al.*, 1994).

Several antibodies have been described that bind the extracellular part of the α- or β-chain of LFA-1 and are capable of inducing an active conformation of LFA-1, resulting in increased ligand binding (Keizer *et al.*, 1988; Robinson *et al.*, 1992; Andrew *et al.*, 1993; Landis *et al.*, 1993). It is believed that these anti-LFA-1-activating mAbs mimic ligand binding and stimulate post-ligand-binding signaling (outside-in signaling). Outside-in signaling generates different intracellular signals, including phosphorylation of distinct tyrosine kinases and other proteins (Kanner *et al.*, 1993; Arroyo *et al.*, 1994).

Previous experiments have demonstrated that integrins can associate with cytoskeletal components (α-actinin, talin) upon activation (Burn *et al.*, 1988; Pavalko and LaRoche, 1993). Inhibitors such as cytochalasin B and D have been used to inhibit β_2- and β_1-mediated adhesion to their ligands (Rothlein and Springer, 1986; Haverstick *et al.*, 1992; Pyszniak *et al.*, 1994). In contrast, it has recently been reported that cytochalasins can also induce β_2-mediated function (Ross *et al.*, 1992; Elemer and Edgington, 1994; Kucik *et al.*, 1996), whereas others showed no effect from these inhibitors (Diamond and Springer, 1994; Pyszniak *et al.*, 1994). Peter *et al.* (1995) showed that the β_2 cytoplasmic domain was involved in the localization of integrins to focal adhesions and in the organization of the actin cytoskeleton into stress fibers (Peter and O'Toole, 1995). Truncation of the cytoplasmic domain of the β_2 subunit eliminates LFA-1 binding to ICAM-1, indicating that the cytoplasmic domain of β_2 controls adhesiveness (Hibbs *et al.*, 1991a,b). It has been suggested that the reduced adhesiveness of LFA-1 (by deletion of the TTT motif in the cytoplasmic domain of β_2) is due to the altered association/organization of the cytoskeleton rather than a change in the affinity of LFA-1 (Peter and O'Toole, 1995). Since the importance of the distribution (clustered/dispersed) of LFA-1 at the cell surface and its attachment to the cytoskeleton became apparent only recently (van Kooyk *et al.*, 1994; Lub *et al.*, 1995), we investigated the role of the cytoskeleton in the activation of LFA-1 when expressed in leukocytes and nonleukocytes. Therefore, we investigated the distribution and the adhesive capacity of LFA-1 on different cell types in the presence or absence of affinity modulators of LFA-1 (PMA) and cytoskeletal inhibitors. Here, we demonstrate that disruption of the actin cytoskeleton can inhibit or induce LFA-1-mediated adhesion to ICAM-1, depending on the surface distribution of LFA-1 (clustered/dispersed), but independent of the cell type (leukocytes versus nonleukocytes).

MATERIALS AND METHODS

mAbs and Chemicals

The mAbs SPV-L7 (IgG1), NKI-L15 (IgG2a), and NKI-L16 (IgG2a) reactive with the α-chain of human LFA-1 (CD11a) were raised as described previously (Keizer *et al.*, 1985, 1988). The nonblocking mAb TS2/4 (IgG1) reactive with the α-chain of human LFA-1 was kindly provided by Dr. E. Martz (Sanchez-Madrid *et al.*, 1982). The blocking mAb 60.3 against the β_2-chain was kindly provided by Dr. Harlan (Beatty *et al.*, 1983), and the anti-β_2 mAb KIM185 (IgG1) was used to activate LFA-1 (Andrew *et al.*, 1993). Other reagents used were PMA (50 nM, Calbiochem, La Jolla, CA), deoxyglucose (50 mM, Sigma Chemical Co., St. Louis, MO), and sodiumazide (10 mM, Merck, Hohenbrunn, Germany) to deprive the cell from energy, acrylamide (4 mM, Bio-Rad Laboratories, Hercules, CA) to block intermediate filaments, and nocodazole (10 μg/ml, Sigma Chemical Co.) to block microtubules.

Cells and Cell Lines

A homogenous population of highly purified resting T lymphocytes was isolated from buffy coats of healthy donors by centrifugal elutriation, as described previously (Figdor *et al.*, 1981). Activated T lymphocytes were prepared by culturing these cells with recombinant interleukin- (IL) 2 (400 U/ml, Cetus Corp., Emeryville, CA) and phytohemagglutinin (PHA, 0.2 μg/ml, Murex Diagnostics, Dartford, England) in Iscove's medium (Life Technologies, Paisley, Scotland) supplemented with 5% fetal calf serum (BioWhittaker, Verviers, Belgium) and 1% antibiotics/antimycotics (Life Technologies) for 6 days.

L-LFA-1 and BLM-LFA-1 cells were obtained by transfection of the complete cDNA of α_L (in pCDM8) and β_2 (in pRC-CMV containing a neomycin resistance gene, Invitrogen Corporation, San Diego, CA) into mouse fibroblast L cells and human melanoma cells (BLM; Katano *et al.*, 1984) by Ca_2PO_4 precipitation (calcium phosphate transfection system, Life Technologies). After 24 h, the cells were maintained in Iscove's medium containing 5% fetal calf serum, 1% antibiotics/antimycotics, and the neomycin analogue, geneticin (2 mg/ml, Life Technologies). The different transfectants were sorted three times to obtain a homogenous population of cells

expressing high levels of LFA-1. Positive cells were stained directly with fluorescein isothiocyanate (FITC)-labeled TS2/4 mAb. Cells were sorted using the Coulter Epics Elite (Coulter, Hialeah, FL). For establishment of stable $L16^{hi}$ and $L16^{lo}$ L-LFA-1 transfectants, L cells, transfected with LFA-1, were incubated (30 min, 4°C) in phosphate-buffered saline containing 0.5% bovine serum albumin (BSA, Boehringer Mannheim, Mannheim, Germany) and 1% antibiotics/antimycotics, with mAb NKI-L16 (10 μg/ml), followed by incubation with FITC-labeled goat $(Fab')_2$ anti-mouse IgG mAb (Zymed Laboratories, San Francisco, CA) for 30 min at 4°C. Cells were positively and negatively selected on expression of the L16 epitope by sorting using the Coulter Epics Elite. To obtain a homogenous population of $L16^{lo}$- and $L16^{hi}$-expressing L-LFA-1 cells, the sorting procedure was repeated three times.

Immunofluorescence Analysis

Cells were incubated (30 min, 4°C) in phosphate-buffered saline containing 0.5% wt/vol BSA and 0.01% sodiumazide, with appropriate dilutions of the different mAb, followed by incubation with FITC-labeled goat $(Fab')_2$ anti-mouse IgG mAb for 30 min at 4°C. The relative fluorescence intensity was measured by FACScan analysis (Becton Dickinson, Oxnard, CA).

Cell Adhesion Assays

Binding of LFA-1-positive cells to ICAM-1 was performed using ICAM-1 fusion proteins consisting of the five domains of ICAM-1 fused to a human IgG1 Fc fragment (ICAM-1Fc). Culture supernatant of mouse fibroblast cells (L-cells) transfected with pICAM-1Fc cDNA (Fawcett *et al.*, 1992) was tested for the presence of ICAM-1Fc in an IgG-specific enzyme-linked immunosorbent assay. To coat ICAM-1 Fc, 50 μl of goat anti-human Fc-specific $F(ab')_2$ (4 μg/ml, Jackson ImmunoResearch Laboratories, Westgrove, PA) were precoated on 96-wells flat-bottomed plates (MaxiSorp, Nunc, Roskilde, Denmark) for 1 h at 37°C. Subsequently, wells were blocked by 1% BSA in TSM (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) for 30 min at 37°C. Culture supernatant of ICAM-1Fc (approximately 40 ng/ml) was coated for 1 h at 37°C. After labeling of the cells with $Na_2{}^{51}CrO_4$ (Amersham International, Buckinghamshire, England) for 1 h at 37°C, they were preincubated with cytochalasin D (5 μg/ml) in the presence or absence of either deoxyglucose (50 mM) and sodiumazide (10 mM) or acrylamide (4 mM) and nocodazole (10 μg/ml) for 30 min at 37°C. Radiolabeled cells were preincubated for 10 min at room temperature with different stimuli and/or blocking mAbs. Cells were allowed to adhere for 30 to 40 min at 37°C. Unbound cells were removed by washing with TSM supplemented with 0.5% wt/vol BSA. The adherent cells were lysed with 100 μl of 2% Triton X-100, and radioactivity was quantified in a gamma counter. Results are expressed as the mean percentage of cells binding from triplicate wells. Values are depicted as LFA-1-specific adhesion: Percentage of cells binding − percentage of cells binding in the presence of an integrin blocking mAb (NKI-L15, 10 μg/ml).

Confocal Microscopy

Adherent cells were grown overnight (adherent cells: BLM and L cell transfectants) on glass slides and nonadherent cells [resting peripheral blood lymphocytes (PBLs) and activated PBLs] were immobilized on glass slides precoated with Celltak adhesive (Collaborative Biomedical Products, Bedford, MA). Cells were pretreated with cytochalasin D (5 μg/ml; 10 min at 37°C) and activated with PMA (50 nM) for 20 min at 37°C in the presence of cytochalasin D, followed by fixation with 0.5% paraformaldehyde. Fixed cells were stained with the mAb TS2/4 (10 μg/ml) for 30 min at 37°C, followed by incubation with FITC-labeled goat $(Fab')_2$ anti-mouse IgG mAb for 30 min at room temperature. Cell surface distribution of integrins was determined by confocal laser scanning microscopy (CLSM) at 488 nm with a krypton/argon laser (Bio-Rad). In all experiments, the same instrument settings of the CLSM were used.

RESULTS

Expression of LFA-1 by L Cell Fibroblasts and BLM Melanoma Cells

To compare the function of LFA-1 in leukocytes with that in nonleukocytes, we generated transfectants in which both the α and β subunit of LFA-1 were transfected. We expressed LFA-1 in murine fibroblast cells (L cells) and human melanoma cells (BLM). Expression level of the α-chain of LFA-1 was similar to that on resting and IL-2/PHA-activated PBLs (Figure 1). Expression of the β-chain of LFA-1 was higher on activated PBLs compared with resting PBLs, which is caused by expression of Mac-1 and p150.95, other members of the β_2 integrin subfamily, on activated PBLs (Figure 1).

Since multimerization or clustering of LFA-1 (high avidity) facilitates binding to ICAM-1, we investigated expression of the L16 epitope, which reports the clustered status of LFA-1 on the cell surface of hematopoietic cells (van Kooyk *et al.*, 1994). Staining LFA-1 with mAb NKI-L16 reveals distinct expression patterns of this epitope, depending on the cell type. Both BLM-LFA-1 and L-LFA-1 transfectants show an L16 expression, which equals the expression of LFA-1 obtained with a regular mAb SPV-L7 (NKI-L16:SPV-L7 peak channel ratio = 1, ranging from 0.9 to 1.2) and is comparable to activated PBLs. In contrast, LFA-1 on resting PBLs expresses significantly less of the L16 epitope (NKI-L16:SPV-L7 peak channel ratio = 0.2, ranging from 0.1 to 0.3). This indicates that LFA-1, on BLM, L cell transfectants, and activated PBLs, is constitutively expressed in clusters on the cell surface.

To prove that L16 expression on these transfectants indeed correlates with LFA-1 clustering as it does on leukocytes, we analyzed the surface distribution of LFA-1 on the L-LFA-1 and BLM-LFA-1 transfectants by confocal microscopy and compared it with the distribution of LFA-1 on resting and IL-2/PHA-activated T cells (Figure 2). The two LFA-1 transfectants that express high levels of the L16 epitope (Figure 2, A, L-LFA-1; and B, BLM-LFA-1) show a clustered distribution of LFA-1 on the cell surface similar to IL-2/PHA-activated PBLs (Figure 2C). In contrast, resting PBLs show a dispersed distribution of LFA-1 (Figure 2D), which corresponds with the low L16 expression on these cells. In conclusion, also in nonhematopoietic cells, expression of the L16 epitope correlates with a clustered distribution of LFA-1.

Clustering of LFA-1 Facilitates ICAM-1 Binding

Since the various LFA-1 transfectants exhibited major differences in LFA-1 distribution, we investigated the

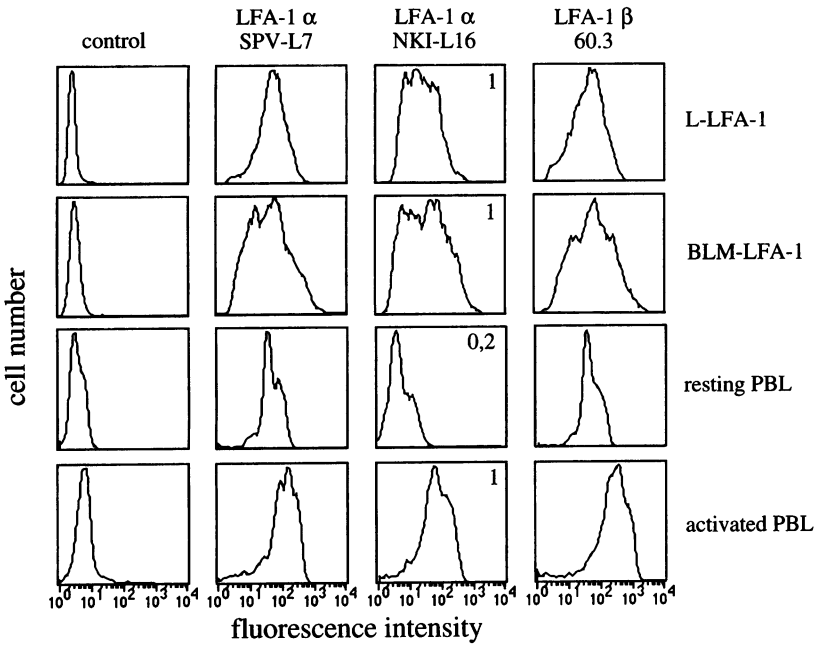

Figure 1. Expression of LFA-1 and the LFA-1 activation epitope (L16) on distinct cells. L-LFA-1, BLM-LFA-1, and resting and IL-2/PHA-activated PBLs were stained with either isotype-matched control antibodies or specific antibodies directed against the α subunit of LFA-1 (SPV-L7) and (NKI-L16) or the β-chain of LFA-1 (60.3) and GAM-$(Fab')_2$-FITC second antibodies. NKI-L16:SPV-L7 ratio is mentioned in the NKI-L16 histogram. One of five experiments is shown.

intrinsic activation state of the receptor by measuring LFA-1-mediated adhesion to ICAM-1 without the addition of any activating stimuli. The results in Figure 3 demonstrate that BLM-LFA-1-, L-LFA-1-, and IL-2/PHA-activated PBLs spontaneously bind to ICAM-1. In contrast, resting PBLs that show a dispersed LFA-1 distribution hardly bind to ICAM-1 in the absence of stimuli (Figure 3). The binding to ICAM-1 is LFA-1 specific and could not be attributed to Mac-1 or p150.95 expression, since the depicted specific binding was determined in the presence of a blocking anti-LFA-1α mAb. The difference in the spontaneous adhesion of these cells to ICAM-1 is not due to distinct levels of LFA-1 expression, since all cells have similar levels of LFA-1 expression (SPV-L7), but only differ in

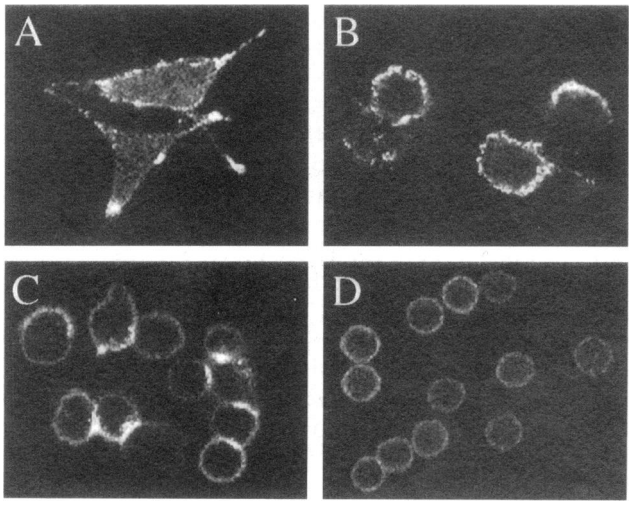

Figure 2. Surface distribution of LFA-1 expressed as determined by CLSM using a FITC-labeled anti-LFA-1 mAb TS2/4. LFA-1 is localized in large clusters on L-LFA-1 (A), BLM-LFA-1 (B), and on IL-2/PHA-activated PBLs (C), whereas it is dispersed on resting PBLs (D). The instrument settings of the CLSM were the same for the distinct photographs.

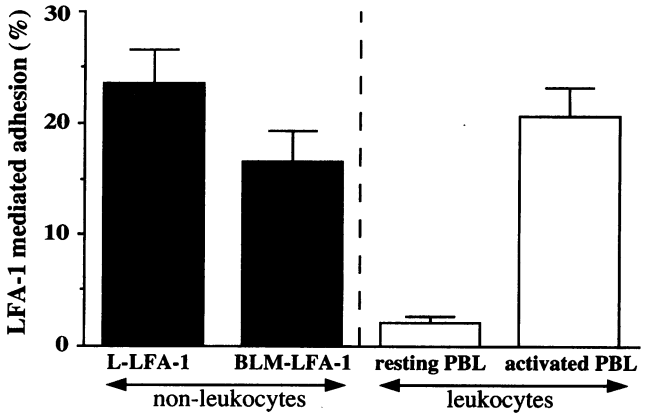

Figure 3. Spontaneous LFA-1-mediated adhesion to ICAM-1. L-LFA-1, BLM-LFA-1, resting PBLs, and IL-2/PHA-activated PBLs were allowed to adhere to chimeric ICAM-1Fc-coated wells for 30 min at 37°C. Depicted is the mean percentage of LFA-1-specific adhesion to ICAM-1 of triplicate wells. Specific adhesion is the percentage of cells binding − the percentage of cells binding in the presence of an LFA-1 blocking mAb (NKI-L15). Adhesion which could not be blocked by NKI-L15 was always less than 5% on activated PBLs. One representative experiment of five is shown.

expression of the L16 epitope (Figure 1). These data indicate that clustering of LFA-1 on the cell surface induces LFA-1/ICAM-1 binding (van Kooyk *et al.*, 1994).

Since binding to ICAM-1, which alters the affinity state of LFA-1, can also be enhanced upon activation of cells with PMA (Lollo *et al.*, 1993), we activate the cells with PMA and the activating anti-LFA-1β mAb KIM185 which both induce an active conformation (high-affinity state) of LFA-1. The results in Figure 4A show that binding of resting PBLs to ICAM-1 indeed requires additional stimuli, like KIM185 or PMA. In contrast, adhesion of IL-2/PHA-activated PBLs, BLM-LFA-1, and L-LFA-1 to ICAM-1, which bind already spontaneously to ICAM-1 (Figure 3), can be further increased by PMA or the activating mAb KIM185 (Figure 4, B–D). However, PMA is significantly less effective in inducing LFA-1-mediated adhesion to ICAM-1 of resting PBLs (Figure 4A) when compared with IL-2/PHA-activated PBLs (Figure 4B), which is in line with previous findings (van Kooyk *et al.*, 1993, 1994). Thus, when LFA-1 is expressed on either hematopoietic or nonhematopoietic cells, adhesion to ICAM-1 can be induced by alteration in avidity (clustering) or affinity (induced by PMA or KIM185), whereas both avidity and affinity alteration cooperate to induce maximal adhesion.

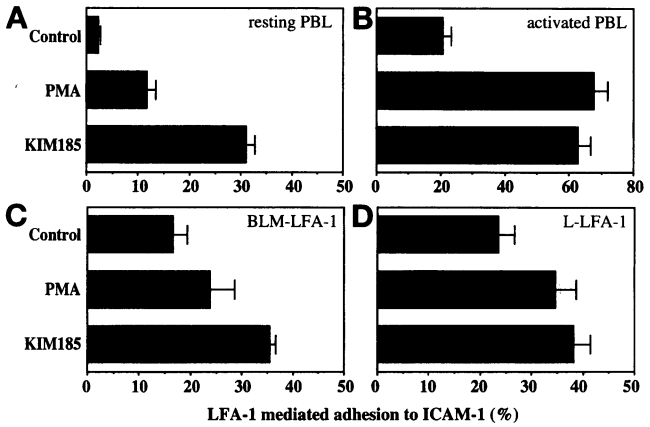

Figure 4. Induction of LFA-1-mediated adhesion to ICAM-1 of adherent transfectants and leukocytes by different LFA-1-activating stimuli. Resting PBL cells (A), IL-2/PHA-activated PBLs (B), BLM-LFA-1 (C), and L-LFA-1 (D) were preincubated in medium (control), PMA (50 nM), or the activating antibody KIM185 (5 μg/ml). Depicted is the mean percentage of LFA-1-specific binding to ICAM-1 of three independent wells. Specific adhesion is the percentage of cells binding − the percentage of cells binding in the presence of an LFA-1 blocking mAb (NKI-L15). Adhesion which could not be blocked by NKI-L15 was always less than 5% on IL-2/PHA-activated PBLs. Data are representative of three experiments.

Dual Role of Filamentous Actin (F-Actin) in Regulating LFA-1 Function

The importance of the association of cytoskeletal components (α-actinin and talin) with the β_2-chain has been reported previously (Burn *et al.*, 1988; Pavalko and LaRoche, 1993). However, it is not known whether association with the cytoskeleton is important in modulating the affinity, the distribution of LFA-1, or both. We therefore investigated the role of the actin cytoskeleton in LFA-1-mediated adhesion to ICAM-1 by different cell types, which either show a dispersed (resting PBLs) or a clustered LFA-1 distribution (IL-2/PHA-activated PBLs) on the cell surface.

Interestingly, when actin polymerization is inhibited in resting or in IL-2/PHA-activated T cells, clear differences are observed. Treatment of resting PBLs (dispersed LFA-1 distribution) with cytochalasin D or PMA increases adhesion of LFA-1 to ICAM-1. However, cytochalasin D treatment followed by activation of the cells with PMA results in more than a twofold increase in LFA-1-mediated adhesion to ICAM-1 compared with untreated, PMA-stimulated cells (Figure 5A), suggesting that both uncoupling of LFA-1 from the cytoskeleton and induction of an active conformation are necessary to achieve strong adhesion. In contrast, treatment of IL-2/PHA-activated PBLs (clustered LFA-1 distribution) with cytochalasin D results in a decreased LFA-1-mediated adhesion to ICAM-1, with or without the activation of LFA-1 with PMA (Figure 5B).

Since on K562 cells transfected with a β_2 cytoplasmic deletion mutant of LFA-1, LFA-1 is localized in large clusters and cytochalasin D uncouples actin from integrin molecules, the enhanced motility of LFA-1 at the cell surface may allow cluster formation that results in an increased adhesion to ICAM-1, we hypothesize that the cytoskeleton affects the distribution of

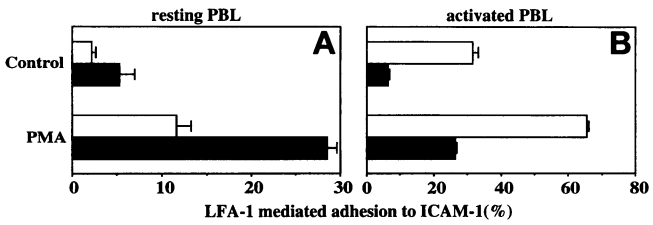

Figure 5. Role of F-actin in regulating LFA-1-mediated adhesion. LFA-1-mediated adhesion to ICAM-1 of cytochalasin D-treated (■) or untreated cells (□). PBLs (A) and IL-2/PHA-activated PBLs (B) were first treated with cytochalasin D (5 μg/ml, 30 min at 37°C) or untreated as a control, followed by preincubation with PMA (50 nM). The mean percentage of LFA-1-specific adhesion of triplicate wells is shown. Specific adhesion is the percentage of cells binding − the percentage of cells binding in the presence of a LFA-1 blocking mAb (NKI-L15). Adhesion which could not be blocked by NKI-L15 was always less than 5% on IL-2/PHA-activated PBLs. One representative experiment of three is shown.

LFA-1. To test this hypothesis, resting PBLs were pretreated with cytochalasin D and stained with an anti-LFA-1α mAb (Figure 6). Figure 6A shows the diffuse LFA-1 cell surface distribution on untreated resting PBLs, whereas Figure 6, C and D, demonstrate that cytochalasin D treatment of these cells results in a clustered LFA-1 distribution more similar to the clustered LFA-1 distribution observed on IL-2/PHA-activated PBLs (Figures 2C and 7A). Together with the adhesion data, this suggests that cytochalasin D induces LFA-1/ICAM-1 adhesion by increasing the avidity (clustering) of LFA-1 on resting PBLs. PMA activation itself has no effect on the cell surface distribution of LFA-1 on untreated cells (Figure 6B) or on cytochalasin D-treated cells (Figure 6D), indicating that PMA alters the affinity rather than the avidity of LFA-1. In contrast, treatment of IL-2/PHA-activated PBLs with cytochalasin D blocks the adhesion of these cells to ICAM-1 (Figure 5B). Cytochalasin D does not seem to affect the clustered distribution of LFA-1 on the cell surface (Figure 7,C and D), as measured by CLSM. However, we cannot exclude that cytochalasin D also affects to some extent the distribution of LFA-1 on IL-2/PHA-activated PBLs, since minor differences in preexisting clusters cannot be distinguished by CLSM. PMA activation has no effect on the LFA-1 distribution of IL-2/PHA-activated PBLs (Figure 7, B and D), whereas it has been shown to enhance adhesion of these cells to ICAM-1 (Figure 5B). Together with recent findings (Kucik *et al.*, 1996), this suggests that PMA alters the affinity of LFA-1, whereas cytochalasin D affects the avidity of LFA-1.

To summarize our findings (Figure 8) on resting PBLs, LFA-1 is homogenously distributed and hardly binds ICAM-1. Only the combination of PMA, to induce a high-affinity receptor, and cytochalasin D treatment results in a clustered LFA-1 cell surface distribution and strong ICAM-1 binding (30%). On IL-2/PHA-activated PBLs, LFA-1 is already found in macro clusters and binds ICAM-1 in the absence of any stimulus (30%). PMA activation of IL-2/PHA-activated PBLs can increase the affinity of LFA-1 for ICAM-1 binding (67%). Inhibition of the actin polymerization in these cells decreases adhesion to ICAM-1 to the level of the nonstimulated IL-2/PHA-activated PBLs, probably by decreasing the major clustering of LFA-1 on these cells, which we cannot distinguish with CLSM (see DISCUSSION). These data suggest that the actin cytoskeleton is involved in two steps of the activation process of LFA-1: it maintains LFA-1 in a homogenously distributed (low-avidity) state on resting PBLs, and the actin cytoskeleton maintains LFA-1 in a high-avidity state (clustered) on activated PBLs.

Dual Role of F-Actin in LFA-1-mediated Adhesion Is not Restricted to Leukocytes

To investigate whether the dual role of F-actin in regulating LFA-1 function is restricted to leukocytes,

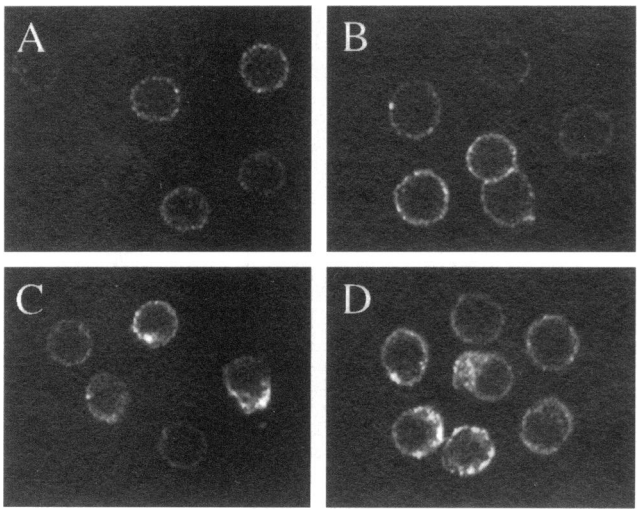

Figure 6. LFA-1 distribution of cytochalasin D-treated resting PBLs shifts toward a clustered distribution of LFA-1. Resting PBLs were pretreated with cytochalasin D (5 μg/ml, C and D) or as a control untreated (A and B) and were either activated with PMA (B and D) or nonactivated (A and C). All cells were stained for 30 min with the anti-LFA-1 mAb TS2/4 (10 μg/ml) and FITC-labeled goat $(Fab')_2$ anti-mouse IgG. Fluorescence distribution was determined with CLSM at 488 nm. The instrument settings of the CLSM were the same for the distinct panels.

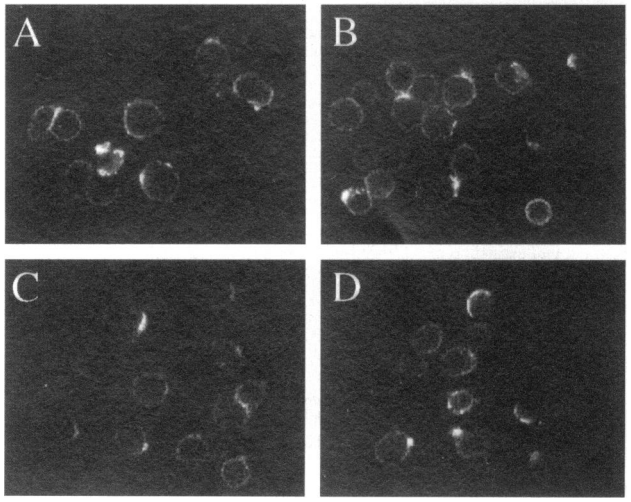

Figure 7. Cytochalasin D does not affect the LFA-1 distribution on IL-2/PHA-activated PBLs. IL-2/PHA-activated PBLs were pretreated with cytochalasin D (5 μg/ml; C and D) or as a control untreated (A and B) and were either activated with PMA (B and D) or nonactivated (A and C). All cells were stained for 30 min with the anti-LFA-1 mAb TS2/4 (10 μg/ml) and FITC-labeled goat $(Fab')_2$ anti-mouse IgG. Fluorescence distribution was determined by CLSM at 488 nm. The same instrument settings of the CLSM were used throughout the experiment.

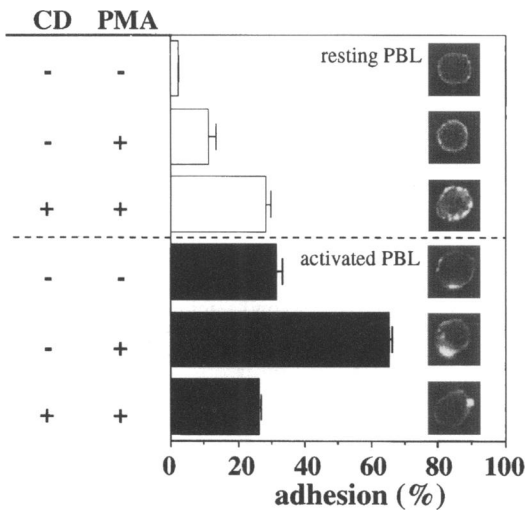

Figure 8. The dual role of F-actin in regulating LFA-1-mediated adhesion and its correlation with the distribution of LFA-1 on both resting and IL-2/PHA-activated PBLs. This figure integrates the adhesion data presented in Figure 5 with the distribution data shown in Figures 6 and 7. Cytochalasin D (CD) induces LFA-1 clustering and ICAM-1 adhesion on resting PBLs, whereas it does not affect the LFA-1 distribution, but inhibits the binding to ICAM-1 of IL-2/PHA-activated PBLs. Adhesion which could not be blocked by NKI-L15 was always less than 5% on IL-2/PHA-activated PBLs.

we determined the effect of cytochalasin D on the adhesion to ICAM-1 of two distinct L-LFA-1 transfectants. We therefore selected L-LFA-1 cells that have a low expression of the L16 epitope (Figure 9; NKI-L16: SPV-L7 peak channel ratio of approximately 0.1) in contrast to the normal L-LFA-1 transfectants that express high levels of the L16 epitope (Figure 9; NKI-L16:SPV-L7 peak channel ratio of approximately 1). The $L16^{lo}$ (resting) L-LFA-1 transfectants show increased adhesion to ICAM-1 upon preincubation with cytochalasin D and stimulation with PMA (Figure 10), whereas the adhesion of $L16^{hi}$ (activated) L-LFA-1 cells is inhibited under the same conditions (Figure 10). This indicates that the dual role of F-actin in regulating LFA-1 function is independent on the cell type on which LFA-1 is expressed.

Intact Intermediate Filaments, Microtubules, and Energy Are Essential in LFA-1/ICAM-1 Binding of Cytochalasin D-treated Cells

To determine whether induction of adhesion by cytochalasin D is an active, energy-dependent process, we treated resting $L16^{lo}$-expressing L-LFA-1 cells with sodiumazide and deoxyglucose. Our unpublished results demonstrated that sodiumazide or deoxyglucose alone was not effective in blocking the cytochalasin D-induced adhesion, whereas a combination of both inhibited adhesion, indicating that induction of adhesion caused by cytochalasin D is energy dependent (Figure 11). To analyze whether other cytoskeletal proteins participate in the cytochalasin D-induced adhesion of $L16^{lo}$ L-LFA-1 cells to ICAM-1, we treated the cells with acrylamide and nocodazole, disrupting the intermediate filaments and microtubules, respectively. Neither nocodazole nor acrylamide alone was effective in inhibiting adhesion, whereas Figure 11 demonstrates that the induced adhesion of cytochalasin D-treated cells is inhibited by a combination of acrylamide and nocodazole. This indicates that both intermediate filaments and microtubules are essential to mediate this adhesion to ICAM-1. Our unpublished observations demonstrated that disruption of the intermediate filaments or microtubules in cells that are $L16^{hi}$ did not inhibit LFA-1 mediate adhesion to ICAM-1.

DISCUSSION

We have demonstrated the following: 1) On resting leukocytes, LFA-1 is homogenously distributed, whereas on IL-2/PHA-activated PBLs LFA-1 is localized in clusters on the cell surface. 2) On cells with a clustered LFA-1 phenotype, association of LFA-1 with the actin cytoskeleton is essential for strong adhesion to ICAM-1. 3) Uncoupling F-actin from LFA-1 (by cytochalasin D) induces LFA-1-mediated binding to ICAM-1 of cells that have a homogenous LFA-1 surface distribution by facilitating lateral movement of LFA-1 into clusters. 4) This cytochalasin D-induced adhesion to ICAM-1 is dependent on both intact intermediate filaments and microtubules and is energy dependent.

On resting PBLs, LFA-1 is homogenously distributed and hardly binds ICAM-1. In contrast, on IL-2/PHA-activated PBLs and L-LFA-1 and BLM-LFA-1 transfectants, LFA-1 is localized in huge clusters and spontaneously binds ICAM-1 (approximately 20–30%). In addition, adhesion of these cells can be increased by PMA or the activating mAb KIM185, demonstrating that besides clustering of LFA-1 (avidity), affinity changes in the LFA-1 molecule (active conformation) are still required for strong binding to ICAM-1. These findings, along with data that integrins can associate with cytoskeletal components (α-actinin and talin), in particular through the β-chain (Burn *et al.*, 1988; Pavalko and LaRoche, 1993), led us to hypothesize that certain cytoskeletal proteins are essential to maintain LFA-1 in a high-avidity state on these cells. The observation that cytochalasin D, which inhibits actin polymerization, blocks the adhesion (in the absence of an activator of LFA-1) of the L cell, BLM transfectants, and IL-2/PHA-activated PBLs supports this hypothesis and demonstrates that this phenomenon is not leukocyte restricted. In contrast, on resting PBLs and L-LFA-1/$L16^{lo}$, detachment of the distributed homogenous LFA-1 from the actin cytoskeleton,

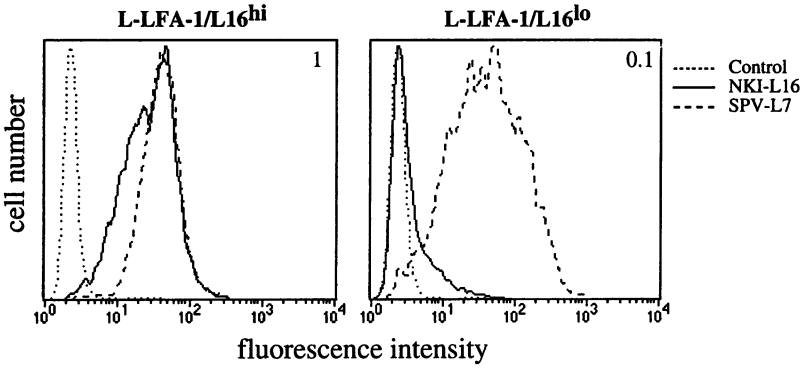

Figure 9. Expression of the LFA-1 activation epitope (L16) on $L16^{hi}$ and $L16^{lo}$ L-LFA-1 transfectants. $L16^{hi}$ and $L16^{lo}$ L-LFA-1 transfectants were stained with either isotype-matched control antibodies or specific antibodies directed against the α subunit of LFA-1 (SPV-L7 and NKI-L16) and GAM-$(Fab')_2$-FITC second antibodies. The NKI-L16: SPV-L7 ratio of both transfectants is mentioned in the histograms. One of five experiments is shown.

by cytochalasin D, may facilitate redistribution of LFA-1 into clusters (Figure 6; Lub *et al.*, 1995). Cells with this high-avidity form of LFA-1 (clustered) will bind better to ICAM-1 than cells on which LFA-1 is homogenously distributed, thus explaining the enhanced adhesion when these cells are treated with cytochalasin D. On IL-2/PHA-activated PBLs, clustering of LFA-1 may be caused by culturing these cells with IL-2 and PHA, since it is possible that IL-2/PHA activates LFA-1 to interact with ICAM-1 on the opposite cell, thereby causing cytoskeletal reorganizations and clustering of LFA-1 on these cells, as has been suggested recently for the $\alpha_{IIb}\beta_3$ integrin (Fox *et al.*, 1996). The observation that PMA is required demonstrates that clustering of LFA-1 in itself is essential but not sufficient to stimulate strong adhesion. Both a high-avidity (clustering) and high-affinity state (active conformation) of LFA-1 cooperate for strong adhesion. Our unpublished results demonstrate that in contrast to LFA-1, on both resting and IL-2/PHA-activated PBLs, β_1 integrin-mediated adhesion is always inhibited when actin polymerization is inhibited, indicating that β_1 and β_2 integrins differ in their coupling to the cytoskeleton.

Figure 12 explains the dual role of the actin cytoskeleton in the activation of LFA-1 in leukocytes. In this model the correlation between adhesion capacity and cell surface distribution of LFA-1 on leukocytes is depicted. When LFA-1 is inactive, as on resting PBLs, it has a dispersed distribution and actin filaments keep the LFA-1 molecules in a low-avidity state (repression). Temporarily uncoupling of LFA-1 from the cytoskeleton by PMA is necessary to allow conformational changes in the LFA-1 molecule (Kucik *et al.*, 1996). Along with lateral movement of the adhesion receptor by cytochalasin D, this results in the acquisition of clustered (high-avidity) LFA-1 molecules with high ligand-binding affinity (Lub *et al.*, 1995). Recently it has also been shown by others that release from cytoskeletal constraints is an important early step in activation of adhesion, and that the actin cytoskeleton is actively maintaining LFA-1 in its nonadhesive (low-affinity and -avidity) state (Kucik *et al.*, 1996). Furthermore, other cytoskeletal proteins (e.g., intermediate filaments and microtubules) are essential for the induced LFA-1-mediated adhesion of cytochalasin D-treated cells to ICAM-1. The association of LFA-1 with these cytoskeletal proteins may either induce an active conformation of LFA-1, allow lateral movement of LFA-1 on the cell, or a combination of both. More evidence comes from K562 cells transfected with a β_2 cytoplasmic deletion mutant of LFA-1. On these transfectants, LFA-1 is localized in large clusters, suggesting that uncoupling of the β_2 from the cytoskeleton indeed facilitates redistribution of LFA-1 (Lub, van Vliet, Oomen, Pieters, Robinson, and van Kooyk, unpublished data). These data suggest that F-actin is important in maintaining LFA-1 in its low-avidity state on resting PBLs (Figure 12, 1), whereas it is important to maintain LFA-1 in its high-avidity state (clustered) on IL-2/PHA-activated PBLs (Figure 12, 5) and $L16^{hi}$ transfectants. However, we probably cannot distinguish the minor difference in LFA-1 clustering on cytochalasin D-treated resting PBLs (clustering stage II, Figure 12, 3 and 4) and LFA-1 clustering on unstimulated IL-2/PHA-activated PBLs (clustering stage III, Figure 12, 5 and 6), since only major differences in clustering can be distinguished by CLSM (Figure 12; major differences in clustering between stage I and II can be observed with CLSM). The transition from inactive to active LFA-1 molecules is clearly dependent on intermediate filaments and microtubules. In addition, cytochalasin D seems to lower the avidity of LFA-1 on IL-2/PHA-activated PBLs, whereas it increases the avidity of LFA-1 on resting PBLs, suggesting that the association of LFA-1 with the cytoskeleton is different in resting PBLs compared with IL-2/PHA-activated PBLs.

The notion that cytochalasins induce rather than inhibit adhesion is also supported by several recent findings. It has been demonstrated that inhibition of actin polymerization by cytochalasins increases in-

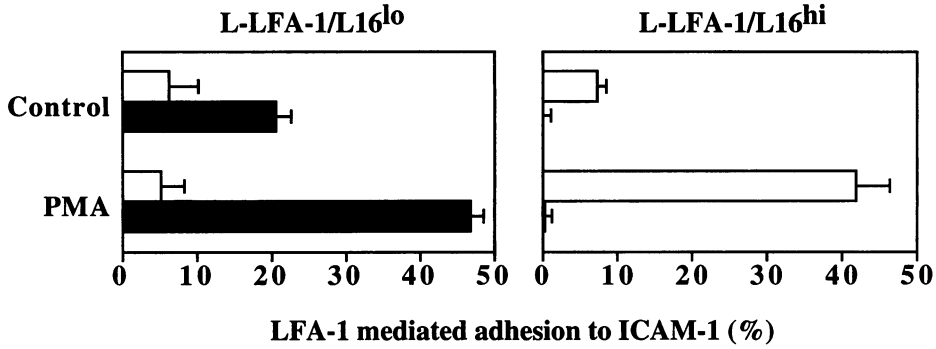

Figure 10. Distinct role of the actin cytoskeleton in LFA-1-mediated ICAM-1 binding of $L16^{lo}$ and $L16^{hi}$ L-LFA-1 transfectants. L-LFA-1 transfectants that show a high L16 expression and L-LFA-1 cells that exhibit a low L16 expression were treated with cytochalasin D (■, 5 μg/ml, 30 min at 37°C) or untreated (□). Depicted is the percentage of LFA-1-mediated adhesion to ICAM-1 of nontreated or cytochalasin D-treated cells in triplicate wells. LFA-1-mediated adhesion is the percentage of cells binding − the percentage of cells binding in the presence of an LFA-1 blocking mAb (NKI-L15). Data are representative of three experiments.

tegrin motility on endothelial cells (Wang *et al.*, 1993). Furthermore, it has been shown (Elemer and Edgington, 1994) that inhibition of actin polymerization enhances ligand binding by Mac-1 on monocytic cells and by LFA-1 on B cells (Kucik *et al.*, 1996). In addition, inhibition of actin polymerization increases the ability of macrophages to form p150.95-dependent C3-opsonized sheep erythrocyte (EC3bi) rosettes (Ross *et al.*, 1992). The opposite effects of cytochalasins, e.g., inhibition (Rothlein and Springer, 1986; Haverstick *et al.*, 1992; Pyszniak *et al.*, 1994), induction (Ross *et al.*, 1992; Elemer and Edgington, 1994; Kucik *et al.*, 1996), or no effect (Diamond and Springer, 1994; Pyszniak *et al.*, 1994) in integrin-mediated adhesion can be explained by the following: 1) The use of the more nonspecific actin polymerization inhibitor cytochalasin B instead of the more specific cytochalasin D occurred. 2) In a large number of the studies, cell aggregation assays were used rather than adhesion assays to purify ICAM-1. 3) The distribution state of the integrins on the cell surface was never investigated.

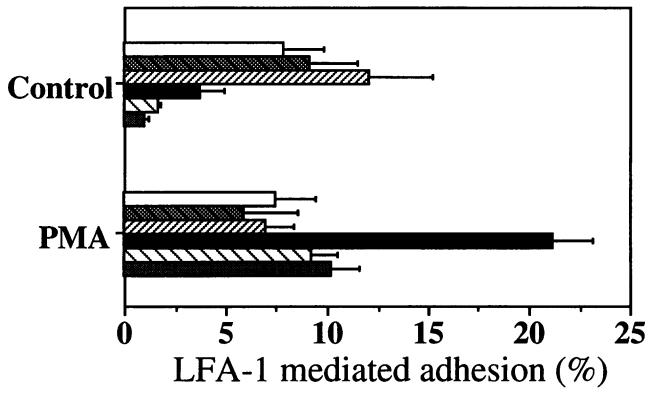

Figure 11. Cytochalasin D-dependent induction of adhesion of $L16^{lo}$ L-LFA-1 cells is dependent on intact intermediate filaments, microtubules, and energy. $L16^{lo}$ L-LFA-1 cells were untreated (□) or treated with metabolic inhibitors: deoxyglucose and sodiumazide (50 mM and 10 mM, respectively, ▧), with inhibitors of the intermediate filaments and microtubules, like acrylamide and nocodazole (4 mM and 10 μg/ml, respectively, ▨), cytochalasin D (5 μg/ml, ■) alone, along with deoxyglucose and sodiumazide (▧), or along with acrylamide and nocodazole (■). Subsequently, cells were activated with PMA with or without the blocking anti-LFA-1 mAb NKI-L15 and allowed to adhere to chimeric ICAM-1Fc for 30 min at 37°C. Depicted is the percentage of LFA-1-specific adhesion to ICAM-1 of nontreated or treated cells in triplicate wells. Specific adhesion is the percentage of cells binding − the percentage of cells binding in the presence of an LFA-1 blocking mAb (NKI-L15). Data are representative of three experiments.

We have demonstrated that coupling of LFA-1 to the actin cytoskeleton is independent of the cell type (leukocytes compared with nonleukocytes). Furthermore, activation of LFA-1 and subsequent ligand binding depends both on the distribution and on the affinity state of the receptor. F-actin has dual functions: 1) to maintain LFA-1 molecules in a clustered form (IL-2/PHA- activated PBLs; $LFA\text{-}1/L16^{hi}$) and 2) to act as a repressor to hold LFA-1 molecules in an inactive form, distributed over the cell surface (resting PBLs; $LFA\text{-}1/L16^{lo}$). We hypothesize that these LFA-1 molecules can only become sensitive to "inside-out" activation when they are temporarily dislodged from the cytoskeleton. Leukocytes require mechanisms to regulate LFA-1 adhesion, since they should be able to circulate as nonadherent cells in blood and lymph and to migrate as adherent cells throughout the tissues. Detachment from and coupling to the actin cytoskeleton provides leukocytes with a mechanism to rapidly alter both the avidity and affinity of LFA-1 to respond to physiological stimuli occurring in vivo.

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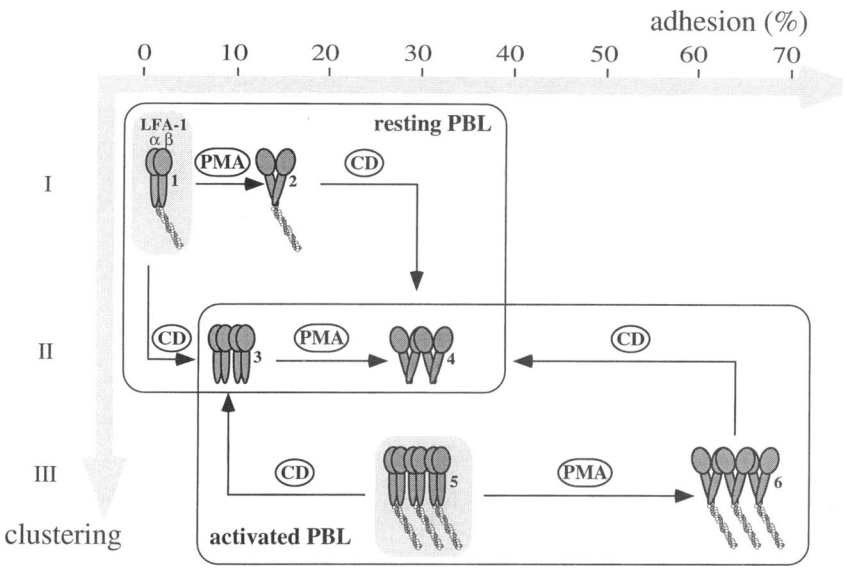

Figure 12. A model that explains the dual role of the actin cytoskeleton in the regulation of LFA-1-mediated adhesion. Depicted is the correlation between adhesion and distribution of LFA-1 on both resting and IL-2/PHA-activated PBLs and the role of the actin cytoskeleton in these processes. On resting PBLs, monomeric LFA-1 receptors are inactive due to connection to the actin cytoskeleton (1). LFA-1 adhesion can be increased by induction of a high-affinity state (active conformation) of LFA-1, through conformational changes, by the phorbol ester PMA (2). On resting PBLs, uncoupling of LFA-1 from the actin cytoskeleton by cytochalasin D (CD) induces clustering (high-avidity state) of LFA-1 and thereby enhances LFA-1/ICAM-1 binding (3). Subsequent PMA stimulation enhances adhesion to ICAM-1 by induction of a high-affinity state (active conformation) of LFA-1 (4). In contrast, on activated PBLs (5), LFA-1 is initially clustered and adhesion can be further increased by enhancing the binding affinity of LFA-1 by PMA (6). Cytochalasin D treatment of IL-2/PHA-activated PBLs partially reduces the avidity (clustering) of LFA-1 (3), resulting in a decreased adhesion to ICAM-1. Clustering of LFA-1 is depicted in different stages (I–III): stage I is no clustering/microclustering of LFA-1 and stage III is macroclustering. Minor differences in clustering between stages II and III cannot be measured with CLSM. Gray boxes indicate the initial state of LFA-1 on resting (1) and IL-2/PHA-activated PBLs (5) prior to PMA or cytochalasin D treatment.

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