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# Signaling Through Integrin LFA-1 Leads to Filamentous Actin Polymerization and Remodeling, Resulting in Enhanced T Cell Adhesion<sup>1</sup>

Joanna C. Porter,\* Madelon Bracke,\* Andrew Smith,\* Derek Davies,<sup>†</sup> and Nancy Hogg<sup>2\*</sup>

The integrins can activate signaling pathways, but the final downstream outcome of these pathways is often unclear. This study analyzes the consequences of signaling events initiated by the interaction of the leukocyte integrin LFA-1 with its ligand, dimeric ICAM-1. We show that the active form of LFA-1 regulates its own function on primary human T cells by directing the remodeling of the F-actin cytoskeleton to strengthen T cell adhesion to ICAM-1. Confocal microscopy revealed that both F-actin bundling and overall levels of F-actin are increased in the ICAM-1-adhering T cells. This increase in F-actin levels and change in F-actin distribution was quantitated for large numbers of T cells using the technique of laser scanning cytometry and was found to be significant. The study went on to show that clustering of conformationally altered LFA-1 is essential for the changes in F-actin, and a model is proposed in which clustered, high-avidity T cell LFA-1, interacting with multivalent ICAM-1, causes LFA-1 signaling, which results in F-actin polymerization and higher-order F-actin bundling. The findings demonstrate that LFA-1 acts not only as an adhesion receptor but also as a signaling receptor by actively initiating the F-actin reorganization that is essential for many T cell-dependent processes. *The Journal of Immunology*, 2002, 168: 6330–6335.

In the fibroblast, integrins are found in focal adhesions connected to prominent bundles of filamentous actin (F-actin),<sup>3</sup> known as stress fibers. This association with cytoskeletal structures is brought about by both integrin cross-linking and ligand binding (1, 2). The clustering of fibroblast integrin  $\alpha_5\beta_1$  also leads to an accumulation of cytoskeletal proteins and 20 signal transduction molecules (1, 3). The interaction of integrins with the cytoskeleton is dynamic, depending on both integrin signaling and the state of the cytoskeletal organization within the cell (reviewed in Ref. 4).

Much less is known about the association in leukocytes of integrins with the cytoskeleton (5). Leukocytes are key migratory cells that relay information to other cells, and their correct functioning depends on successful cell:cell and cell:matrix contacts. For example, the leukocyte integrin, LFA-1, participates in the guided movement of leukocytes from the bloodstream across the vasculature toward the site of injury. LFA-1 also has a key role in the initiation of an immune response by providing adhesion strengthening at the immunological synapse where T cells make contact with APCs (6). The integrins on leukocytes, unlike those on fibroblasts, are constitutively inactive but receive activating stimuli through signaling from other cell surface receptors. Such

receptor-mediated signaling not only will cause clustering of integrins like LFA-1 (7) but will also activate other intracellular signaling pathways. This has made it difficult to distinguish such signals, some of which are necessary for integrin activation, from those that might emanate from the integrin itself upon ligand binding.

In this study we have directly activated LFA-1 by manipulating the extracellular cation environment and have therefore bypassed the usual requirement for an intracellular integrin-activating event. This has allowed the signaling events initiated by integrin ligand binding to be analyzed in isolation from other intracellular signaling pathways (8, 9). The key finding is that the clustering of conformationally altered LFA-1 can independently signal the formation of an F-actin filament network, which is essential for T cell adhesion.

## Materials and Methods

### *Abs, reagents, and cells*

mAb 38 (LFA-1 $\alpha$  subunit, function blocking) was prepared in this laboratory. Other Abs were mAb G25.2 (LFA-1 $\alpha$  subunit, non-function blocking; BD Biosciences, Oxford, U.K.), mAb UCHT1 (CD3; donated by Dr. P. Beverley, Jenner Vaccine Institute, Compton, U.K.), and rabbit anti-mouse-IgG (DAKO, Cambridge, U.K.). The human dimeric five-domain ICAM-1Fc chimeric protein was produced by previously described methods (9). Labels 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein (Calbiochem, Nottingham, U.K.), Alexa Fluor 488-, and TRITC-phalloidin were from Cambridge Biosciences (Cambridge, U.K.). Cytoskeletal inhibitors used were cytochalasin D (Sigma-Aldrich, Cambridge, Dorset, U.K.), latrunculin A (from Dr. R. Treisman, Cancer Research U.K., London, U.K.), and jasplakinolide (Cambridge Biosciences). Primary 10- to 14-day-cultured T lymphoblasts were prepared as previously described (9).

### *Flow cytometry and assessment of soluble dICAM-1 binding*

T cells were washed in assay buffer (20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose (pH 7.4)) and  $2 \times 10^5$  T cells in 50  $\mu$ l assay buffer/0.1% BSA/1–5 mM  $Mg^{2+}$ /1 mM EGTA were added to flexiwell plate wells (Dynex Technologies, Ashford, U.K.) containing 50  $\mu$ l of 2  $\mu$ M dimeric

\*Leukocyte Adhesion Laboratory and <sup>†</sup>Fluorescence Activated Cell Sorter Laboratory, Cancer Research U.K. London Research Institute, London, United Kingdom

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<sup>2</sup> Address correspondence and reprint requests to Dr. Nancy Hogg, Leukocyte Adhesion Laboratory, Cancer Research U.K. London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K. E-mail address: nancy.hogg@cancer.org.uk

<sup>3</sup> Abbreviations used in this paper: F-actin, filamentous actin; dICAM, dimeric ICAM; PLL, poly-L-lysine.

ICAM (dICAM)-1Fc (saturated binding), with or without LFA-1 function-blocking mAb 38 (10  $\mu\text{g}/\text{ml}$ ) or cytochalasin D at a 0.2  $\mu\text{M}$  final concentration. After a 30-min incubation at 37°C, T cells were washed and incubated with 10  $\mu\text{g}/\text{ml}$  FITC-conjugated goat anti-human IgG Fc-specific Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 min on ice. Fluorescence was detected using a FACScan flow cytometer (BD Biosciences).

#### Cell adhesion assays

Flat-bottom Immulon-1 96-well plates (Dynerx Technologies) were pre-coated with 50  $\mu\text{l}$  dICAM-1Fc (3  $\mu\text{g}/\text{ml}$ ) in PBS overnight and blocked with 2.5% BSA. A total of  $2 \times 10^5$  T cells, labeled with 2.5  $\mu\text{M}$  2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein, were treated with either 0–5 mM  $\text{Mg}^{2+}$ /1 mM EGTA or  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (0.4 mM) plus CD3 mAb UCHT1 (10  $\mu\text{g}/\text{ml}$ ) in 100  $\mu\text{l}$  of assay buffer. Cytochalasin D, latrunculin A, and jaspalakinolide, diluted appropriately from stock solutions, were added at the initiation of the experiment. Plates were incubated for 30 min at 37°C. Nonadherent T cells were removed, and adhesion was quantified using a Cytofluor multiwell plate reader series 4000 (PerSeptive Biosystems, Hertford, U.K.) and expressed as a percentage of the total emission before incubation.

#### Confocal microscopy

Thirteen-milliliter round glass coverslips were pre-coated with 400  $\mu\text{l}$  of dICAM-1Fc (3  $\mu\text{g}/\text{ml}$ ) or non-function-blocking LFA-1 mAb G25.2 at 10  $\mu\text{g}/\text{ml}$  overnight at 4°C. Coverslips were blocked with 400  $\mu\text{l}$  of 2.5% BSA in PBS for 1 h at 37°C. Additional coverslips were coated with 400  $\mu\text{l}$  of 0.01% poly-L-lysine (PLL; Sigma-Aldrich) for 5 min followed by washing in RPMI 1640 and allowed to air dry for 2 h.

T cells ( $2 \times 10^5$  per coverslip) were incubated for 30 min at 37°C in 400  $\mu\text{l}$  of assay buffer, including 5 mM  $\text{Mg}^{2+}$  and 1 mM EGTA. In addition, dICAM-1Fc (0.4–2  $\mu\text{M}$ ) was added in solution where indicated. To stain intracellular F-actin, 3% formaldehyde-fixed cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) and incubated with 7.5  $\mu\text{g}/\text{ml}$  TRITC-conjugated phalloidin or 0.18  $\mu\text{g}/\text{ml}$  Alexa Fluor 488-phalloidin for 20 min. Confocal microscopy was performed using a Zeiss laser scanning microscope LSM 510 equipped with a  $\times 63$  oil immersion objective (Zeiss, Oberkochen, Germany). Images were collected as horizontal sections ( $x$ - $y$  plane) taken at 0.5- $\mu\text{m}$  intervals through whole cell volumes and were displayed either as a single mid-cell volume sections or as a projection along the  $z$ -axis. A palette display colored areas of each cell according to total fluorescence from red (brightest fluorescence) to blue (weakest fluorescence).

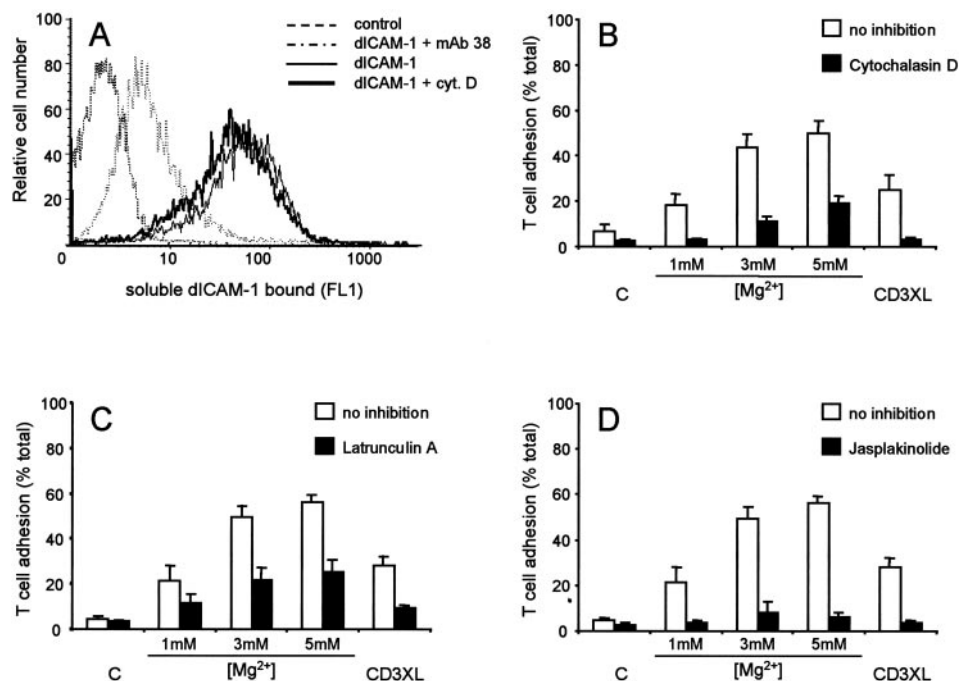
#### Detection of F-actin by laser scanning cytometry

The laser scanning cytometer (CompuCyte, Cambridge, MA) combines features of both flow and image cytometers (10, 11), in that it measures fluorescence and light scatter from immobilized cells that are moved through a 488-nm laser line on a motorized stage. T cells, mounted on coverslips as for confocal microscopy, were defined on the basis of their fluorescence as determined by Alexa Fluor 488-phalloidin-stained F-actin, and the threshold level was optimized so that as many single cells as possible could be contoured without losing fluorescence information. For each cell-contoured event, the following parameters were measured: 1) the area, which represents the physical area in square micrometers occupied by the contoured cell; 2) the integral fluorescence, which is the total amount of fluorescence from all the pixels within a defined cell contour; and 3) the maximum pixel, which is the level of fluorescence of the brightest pixel within the contoured area. Between 5000 and 8000 cells were measured per coverslip. The data were analyzed using an unpaired Student  $t$  test, and a value of  $p < 0.05$  was taken as significant.

## Results

#### Divalent cation treatment of LFA-1 induces direct binding of ligand dICAM-1Fc

Integrins such as LFA-1 require divalent cations for optimal activity. In this study we have taken advantage of the ability of the divalent cation  $\text{Mg}^{2+}$  with EGTA to activate LFA-1-induced adhesion to ligand dICAM-1Fc (8, 9). This procedure brings about direct changes to the LFA-1 ectodomain, enabling higher-avidity binding to soluble dICAM-1Fc. Therefore, T cells that were treated with 5 mM  $\text{Mg}^{2+}$ /1 mM EGTA bound dICAM-1Fc in solution, and this binding was inhibited by the LFA-1-blocking mAb 38 (Fig. 1A). It is relevant to note that the T cells did not express FcRI (mAb 10.1), FcRII (mAb FL18.2), or FcRIII (mAb 3G8) ( $n = 2$ –4; M. Robinson and N. Hogg, unpublished observation), making it unlikely that FcR contributed to the dICAM-1Fc binding. The level of soluble dICAM-1Fc bound to T cells correlated directly with the concentration of  $\text{Mg}^{2+}$  between 1 and 5 mM with no binding in the absence of  $\text{Mg}^{2+}$  (data not shown). Cytochalasin D (2  $\mu\text{M}$ ), an inhibitor of F-actin polymerization, had no effect on binding of soluble dICAM-1Fc (Fig. 1A). This demonstrates that



**FIGURE 1.**  $\text{Mg}^{2+}$ /EGTA-stimulated T cells require an intact cytoskeleton to bind to immobilized but not to soluble dICAM-1Fc. **A**, T cells exposed to 5 mM  $\text{Mg}^{2+}$ /1 mM EGTA or CD3 mAb (10  $\mu\text{g}/\text{ml}$ ) bind 2  $\mu\text{M}$  soluble dICAM-1Fc, which can be blocked by anti-LFA-1 mAb 38 (10  $\mu\text{g}/\text{ml}$ ) but not by 2  $\mu\text{M}$  cytochalasin D. Control, Binding of secondary Ab alone. One experiment representative of three is shown. Adhesion to immobilized dICAM-1Fc is induced by varying concentrations of 0–5 mM  $\text{Mg}^{2+}$ /1 mM EGTA and inhibited by cytochalasin D (**B**, 2  $\mu\text{M}$ ;  $n = 6$ ), latrunculin A (**C**, 1  $\mu\text{M}$ ;  $n = 4$ ), and jaspalakinolide (**D**, 1  $\mu\text{M}$ ;  $n = 4$ ). **C** represents the level of adhesion to ICAM-1 in the absence of  $\text{Mg}^{2+}$ /EGTA.

$Mg^{2+}/EGTA$  directly altered the LFA-1 ectodomain, enabling binding to soluble ligand in a manner that had no dependence on intracellular events associated with the cytoskeleton.

*LFA-1-mediated adhesion to immobilized dICAM-1Fc requires cytoskeletal remodeling*

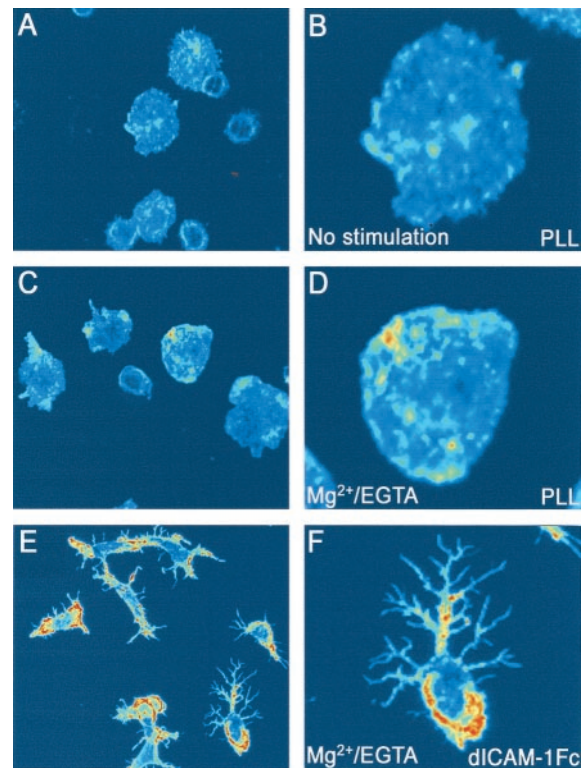
The adhesion of  $Mg^{2+}/EGTA$ -stimulated T cells to immobilized dICAM-1Fc was also detected over a range of  $Mg^{2+}$  concentrations, but adhesion was inhibited by cytochalasin D (Fig. 1B). Therefore, even when LFA-1 is converted to higher-avidity form by exposure to  $Mg^{2+}/EGTA$ , cytoskeletal remodeling is an essential component of T cell adhesion to immobilized dICAM-1Fc. LFA-1-mediated adhesion can also be induced by signaling through receptor complexes, such as TCR/CD3, and, following such “inside-out” signaling, this adhesion was also blocked by cytochalasin D (Fig. 1B) (12, 13). Thus, when signals are delivered both from inside and outside the cell, the cytoskeleton plays a role in LFA-1-mediated T cell adhesion to immobilized ligand.

Cytochalasin D caps the barbed ends of F-actin filaments and prevents their lengthening (14); however, other actin-binding drugs have different modes of action. Both latrunculin A, which blocks polymerization of monomeric G to F-actin (15), and jasplakinolide, which stabilizes preexisting F-actin by inhibiting depolymerization (16), prevented LFA-1-mediated adhesion to dICAM-1Fc (Fig. 1, C and D). The finding that three drugs with distinct effects on the cytoskeleton all interfere with LFA-1-mediated adhesion emphasizes a requirement during the process of adhesion for a dynamic cytoskeleton with necessity for both actin depolymerization and actin repolymerization.

*T cell adhesion to dICAM-1Fc causes F-actin bundle formation*

These results suggested that high-avidity LFA-1, upon interaction with ICAM-1, was able to directly remodel the actin cytoskeleton. Using confocal microscopy, we examined the distribution of F-actin during T cell adhesion. First, control T cells adhered to PLL were of rounded morphology with low levels of F-actin when stimulated either without ( $98.1 \pm 0.1\%$ ;  $n = 3$ ; Fig. 2, A and B) or with  $Mg^{2+}/EGTA$  ( $96 \pm 0.2\%$ ;  $n = 3$ ; Fig. 2, C and D). In contrast,  $Mg^{2+}/EGTA$ -stimulated T cells adherent to dICAM-1Fc had discrete areas with high concentrations of F-actin aggregates or bundles (Fig. 2, E and F). In addition, a majority of T cells ( $75.8 \pm 2.1\%$ ;  $n = 3$ ) had a dramatically altered morphology, with many T cells displaying irregularly arranged filopodia-like processes emanating from the cell body. Thus, LFA-1-mediated adhesion of T cells to immobilized dICAM-1Fc provided the stimulus for F-actin bundle formation and a distinctive shape change.

To provide quantitative validation of the confocal microscopic observations, we used laser scanning cytometry to analyze the F-actin distribution in larger numbers of adherent primary T cells. The same three conditions were compared as in Fig. 2, and the average contoured areas of the adhered cells were  $201 \pm 18 \mu m^2$  (PLL),  $199 \pm 14 \mu m^2$  (PLL/ $Mg^{2+}/EGTA$ ), and  $241 \pm 5 \mu m^2$  (dICAM-1Fc/ $Mg^{2+}/EGTA$ ). Thus, the cells that were spread on dICAM-1Fc covered  $\sim 20\%$  more surface area, in keeping with their elongated shape. To test whether the increase in F-actin bundles, as observed by microscopy, was a general one, the T cells were first compared for the fluorescence levels of localized F-actin (maximum pixel or brightness of F-actin bundles) using laser scanning cytometry. A gate was set to include the majority of the PLL control T cell population (Fig. 3A). When dICAM-1Fc/ $Mg^{2+}/EGTA$ -treated T cells were compared with either the PLL- or PLL/ $Mg^{2+}/EGTA$ -treated cells, a larger proportion had brighter levels of fluorescence ( $35.5$  vs  $7.1$  and  $6.8\%$ , respectively) (Fig. 3, A–C) and a 2-fold overall increase in fluorescence (data not shown).



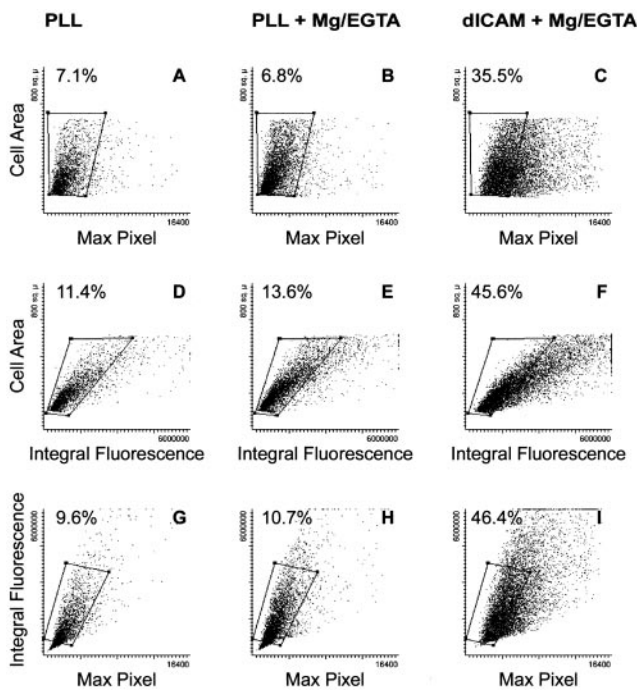
**FIGURE 2.** Confocal microscopic images of F-actin in primary T cells binding to dICAM-1Fc. F-actin was detected with Alexa Fluor 488-phalloidin. A, T cells bound to PLL. B, An enlargement of selected T cell from A. C, T cells bound to PLL and treated with  $Mg^{2+}/EGTA$ . D, An enlargement of selected T cell from C. E, T cells treated with  $Mg^{2+}/EGTA$  and spread on immobilized dICAM-1Fc. F, An enlargement of selected T cell from E. The images represent projections onto the  $x$ - $y$  plane of all individual optical sections taken along the  $z$ -axis using maximal fluorescence values.

Therefore, exposure to immobilized dICAM-1Fc caused new F-actin bundle formation in  $\sim 30\%$  of these primary T cells above the control levels.

To distinguish whether F-actin bundles might derive from de novo F-actin polymerization or from rearrangements of preexisting F-actin, the total or integral amounts of F-actin were compared. Again, a greater proportion of the dICAM-1Fc-adhering T cells had higher levels of F-actin than PLL- and PLL/ $Mg^{2+}/EGTA$ -exposed T cells ( $45.6$  vs  $11.4$  and  $13.6\%$ , respectively; Fig. 3, D–F), indicating an increase in F-actin polymerization for these cells (overall 2-fold increase in arbitrary fluorescence units; data not shown). The close correlation between total fluorescence and maximal brightness of F-actin per T cell (Fig. 3, G–I) suggested that the newly polymerized F-actin was associated with a dynamic conversion to higher-order F-actin bundles and that this process was substantially increased by LFA-1 binding to dICAM-1Fc.

*LFA-1 clustering and high avidity are essential for F-actin bundling and polymerization*

We next investigated those properties of LFA-1 necessary for driving the cytoskeletal rearrangements, in particular whether clustering of LFA-1 was involved. T cells were immobilized with no  $Mg^{2+}$  treatment on PLL or non-function-blocking LFA-1 mAb G25.2 and F-actin levels compared by laser scanning cytometry. Increased levels of both overall fluorescence (integral) and localized fluorescence (maximum pixels) of the F-actin bundles was associated with the mAb G25.2-treated T cells compared with the



**FIGURE 3.** Quantitation of F-actin in T cells using the laser scanning cytometer. The samples of T cells were treated and labeled for F-actin as in Fig. 2; ~7000 cells per sample were analyzed. *A–C*, Scattergram plots of cell area against the maximum pixel (brightness) of F-actin. *A*, T cells bound to PLL. *B*, T cells bound to PLL and stimulated with  $Mg^{2+}/EGTA$ . *C*, T cells stimulated with  $Mg^{2+}/EGTA$  and spread on ICAM-1 have higher levels of localized F-actin compared with *A* and *B*. *D–F*, Scattergram plots of cell area against the integral (total) fluorescence. T cells that are spread on dICAM-1Fc have higher levels of total cellular F-actin compared with the T cells spread on PLL. *G–I*, Scattergram plots of integral fluorescence against maximum pixel. For the T cells spread on dICAM-1Fc, there is correlation between the amount of cellular F-actin and the maximal F-actin brightness or localized F-actin bundles. One experiment representative of three is shown.

PLL-bound T cells (Table I), indicating that LFA-1 clustering was promoting F-actin remodeling. These increases were evident in 23% (total fluorescence) and 17% (maximum pixel) more G25.2 than PLL-tethered T cells, respectively.

Treatment of G25.2-tethered T cells with 5 mM  $Mg^{2+}/1$  mM EGTA, which causes conformational alteration in LFA-1 leading to increased ability to bind dICAM-1Fc (8, 9), caused further enhancement of both F-actin levels and brightness compared with the mAb G25.2 alone. Similarly, a greater proportion of the T cells contained increased levels of F-actin (total fluorescence, 52.9 vs

23%; maximum brightness, 40 vs 17%). There was no significant difference in F-actin associated with the PLL-bound cells  $\pm$  Mg/EGTA. Experiments in which T cells were tethered to immobilized CD5 mAb yielded F-actin levels similar to PLL, indicating that the findings did not depend simply on immobilized mAb (data not shown). Adding soluble dICAM-1Fc in amounts of 0.4  $\mu$ M (or at saturating levels of 2  $\mu$ M; data not shown) to the mAb G25.2- or PLL-tethered T cells treated with  $Mg^{2+}/EGTA$  made no additional difference to the F-actin levels (Table I). The same situation prevailed even when mAb G25.2 was titrated down to levels supporting less than maximum levels of tethering (<1  $\mu$ g/ml) (data not shown).

Next, confocal microscopy was used to compare  $Mg^{2+}/EGTA$ -treated T cells that were tethered on mAb G25.2 or PLL or allowed to bind to dICAM-1Fc. The F-actin content of the mAb G25.2-bound cells resembled the dICAM-1-adhering cells, confirming the laser scanning cytometry results (Fig. 4). Together the findings indicate clustering is essential for the LFA-1-mediated signaling leading to F-actin formation. However, conformationally altered integrin is also required, as tethering of T cells without  $Mg^{2+}/EGTA$  treatment causes much less F-actin polymerization and bundling.

## Discussion

Our results show that LFA-1, the major leukocyte integrin, can act as a true signaling receptor in addition to its role as an adhesion receptor. These signals cause an increase in F-actin associated with cytoskeletal reorganization. It is well described that the adhesion of T cells induced by inside-out signaling can be prevented by cytoskeletal disrupting agents (9, 17). However, it has not previously been recognized that the LFA-1 itself provides signals necessary for cytoskeletal F-actin reorganization and that this is necessary for the adhesion event itself. A key problem has been the difficulty in detecting changes in total F-actin of T cells that are estimated to contain 3.4 pg actin per cell compared with 50 pg in neutrophils (18, 19). Using laser scanning cytometry, consistent increases in total cellular F-actin in the ICAM-1-bound primary T cells can be reliably detected.

The two components of integrin clustering and conformational change have a role in the LFA-1-mediated changes, leading to a maximal increase and remodeling of F-actin. It may be important to provide a framework of clustered active integrin mediating the appropriate signals to build an intracellular F-actin cytoskeletal network. These findings are reminiscent of integrin  $\alpha_5\beta_1$ -mediated signaling in which clustering and exposure of this integrin to ligand mimetic caused F-actin and cytoskeletal-associated proteins to collect around fibronectin-coated beads (1). However, different

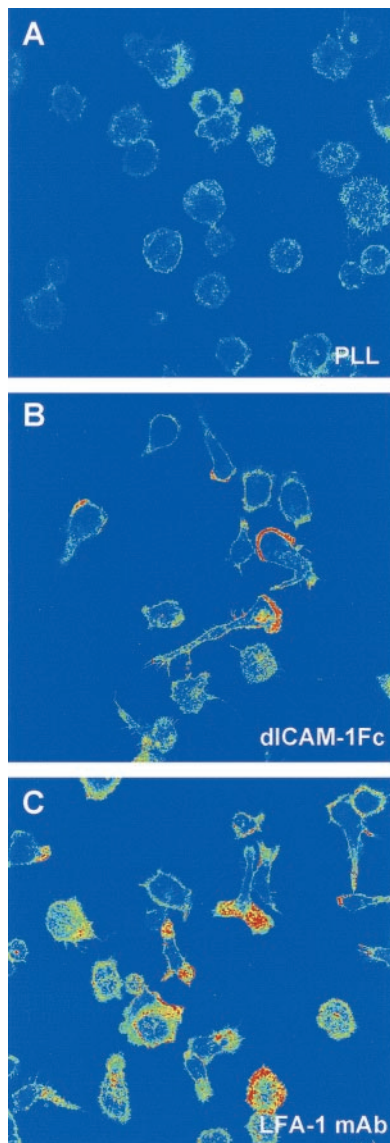
Table I. Quantitation of total and bundled F-actin in T cells following alterations to LFA-1

Treatment	Integral		Maximum Pixel	
	Fluorescence ( $\times 10^3$ )	Cell number <sup>a</sup> (%)	Fluorescence ( $\times 10^3$ )	Cell number <sup>a</sup> (%)
PLL	1105.9 $\pm$ 52.2	14.1 $\pm$ 2.8	2241.7 $\pm$ 151.2	10.0 $\pm$ 1.5
PLL/MgEGTA	1403.4 $\pm$ 193.8	19.9 $\pm$ 6.6 (5.8)	2525.7 $\pm$ 185.4	6.7 $\pm$ 1.6
PLL/MgEGTA/dICAM-1	1316.8 $\pm$ 142.4	16.7 $\pm$ 4.5 (2.6)	2405.3 $\pm$ 107.3	7.3 $\pm$ 0.6
mAb G25.2 (anti-LFA-1)	1499 $\pm$ 17.3 <sup>b</sup>	37.1 $\pm$ 0.3 (23.0)	3471.7 $\pm$ 87.5 <sup>b</sup>	26.7 $\pm$ 2.2 (17)
mAb G25.2/MgEGTA	2011.3 $\pm$ 96.2 <sup>c</sup>	63.3 $\pm$ 3.3 (52.9)	4674.3 $\pm$ 120.1 <sup>c</sup>	49.7 $\pm$ 3.6 (40)
mAb G25.2/MgEGTA/dICAM-1	2065.9 $\pm$ 23.6 <sup>c</sup>	67.0 $\pm$ 1.8 (47.2)	4963.0 $\pm$ 50.2 <sup>c</sup>	51.0 $\pm$ 4.1 (41)

<sup>a</sup> Percentage of total T cells that have fluorescence levels greater than that defined by the gate set to include the majority of the PLL control T cell population. Numbers in parentheses indicate the percentage of T cells expressing increases in F-actin greater than the total PLL-adhering T cell population.

<sup>b</sup> F-actin levels in all mAb G25.2-treated T cells are significantly greater than PLL treated cells ( $p < 0.001$ ).

<sup>c</sup>  $Mg^{2+}/EGTA/G25.2$ -treated T cells have significantly greater amounts of F-actin than T cells tethered on mAb G25.2 alone ( $p < 0.001$ ).



**FIGURE 4.** F-actin assembly at the LFA-1/ICAM-1 interface following stimulation of T cells. Alexa Fluor 488-conjugated phalloidin binding to F-actin in  $Mg^{2+}$ /EGTA-treated T cells adhering to PLL (A), immobilized dICAM-1Fc (B), and anti-LFA-1 mAb G25.2 (C). The images represent projections onto the  $x$ - $y$  plane of all individual optical sections taken at mid-cell volume. One representative experiment of four is shown.

integrins may use alternative mechanisms to connect to the cytoskeletal machinery of the cell. There is evidence that, in fibroblasts, integrin-containing focal adhesions couple to the preformed cytoskeletal network upon contacting ligand (2, 20).

It is interesting that the addition of soluble dICAM-1Fc had no extra effect on the  $Mg^{2+}$ /EGTA-induced high-avidity conformation of LFA-1 in terms of F-actin generation. The presumption is that the presence of excess bound metal ion performs the same role as ligand in either stabilizing or altering integrin conformation. This has been previously demonstrated for both  $Mg^{2+}$ /EGTA (9) and  $Mn^{2+}$  (21) in terms of eliciting expression of integrin activation epitopes. As activated integrin seems instrumental in generating most of the potential F-actin increase, in the situation where F-actin formation occurs in the presence of LFA-1 mAb alone, a smaller reservoir of active LFA-1 receptors may already be expressed by the T cells. In vivo, it is likely that ICAM-1 on the target cell, by interacting with LFA-1, would have a role in pro-

moting these changes in F-actin that we have induced with  $Mg^{2+}$ /EGTA. ICAM-1 is expressed as a dimeric molecule (22, 23) and, on activated endothelium, can be found in leukocyte-induced clusters (24). Thus, ICAM-1 and LFA-1 could promote mutual clustering. Clustering would provide the means of localizing active integrins, thereby increasing the collective signal necessary to generate and remodel the F-actin network.

In this work we have focused on a functional end point of the signaling stimulated by LFA-1. An exciting future prospect will be to determine the individual components of the LFA-1-mediated signaling pathway(s) leading to F-actin formation. LFA-1 has been implicated in the phosphorylation of phospholipase- $\gamma$ 1 (25), focal adhesion kinase, and proline-rich tyrosine kinase-2 (26) and in the protein kinase C  $\beta$ I translocation to the microtubule network (27). These latter kinases are apparently more involved in microtubule than in F-actin reorganization.

In more physiological circumstances, inside-out signals can cause LFA-1 to cluster (7). The cause of the LFA-1 conformational change which leads to affinity increase is less certain, but one study has demonstrated that chemokines cause a transient affinity increase in LFA-1 (28). Thus, signals received by the T cells during an immune response could provide the necessary triggers for both integrin clustering and avidity/affinity changes essential for the F-actin remodeling. The fact that LFA-1 is made active through inside-out signaling suggests that signals from LFA-1 are most effective within the context of activities dictated by other classes of receptor. Thus, signals delivered by chemokines, for example, may direct the spatial distribution of F-actin polymerization observed in chemotaxing leukocytes (29). Another situation is at the immunological synapse formed between T cells and Ag-presenting surfaces, where LFA-1 provides an adhesive contact zone surrounding the Ag-specific TCR (6). It is possible that localized remodeling of the cytoskeleton by LFA-1 at this interface between T cells and target might serve as a scaffold for other signaling molecules or perhaps modulate the movement of other receptors at this dynamic surface. Of course, cytoskeletal remodeling may well occur by signaling through other receptors, such as the TCR (30), although in such studies no account has been taken of a potential contribution from integrins. Finally, the fact that LFA-1 signals to the cytoskeleton in T cells contributes to an evolving awareness of the complexity and specificity of integrin signaling in which these receptors can now claim a much greater role than the purely adhesive.

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## References

- Miyamoto, S., S. K. Akiyama, and K. M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* 267:883.
- Felsenfeld, D. P., D. Choquet, and M. P. Sheetz. 1996. Ligand binding regulates the directed movement of  $\beta_1$  integrins on fibroblasts. *Nature* 383:438.
- Miyamoto, S., H. Teramoto, O. A. Coso, J. S. Gutkind, P. D. Burbelo, S. K. Akiyama, and K. M. Yamada. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* 131:791.
- Horwitz, A. R., and J. T. Parsons. 1999. Cell migration: movin' on. *Science* 286:1102.
- Sanchez-Madrid, F., and M. A. del Pozo. 1999. Leukocyte polarization in cell migration and immune interactions. *EMBO J.* 18:501.
- Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285:221.
- Stewart, M. P., A. McDowall, and N. Hogg. 1998. LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a  $Ca^{2+}$ -dependent protease, calpain. *J. Cell Biol.* 140:699.

8. Dransfield, I., C. Cabanas, A. Craig, and N. Hogg. 1992. Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J. Cell Biol.* 116:219.
9. Stewart, M. P., C. Cabanas, and N. Hogg. 1996. T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. *J. Immunol.* 156:1810.
10. Kametsky, L. A., and L. D. Kametsky. 1991. Microscope-based multiparameter laser scanning cytometer yielding data comparable to flow cytometry data. *Cytometry* 12:381.
11. Darzynkiewicz, Z., E. Bedner, X. Li, W. Gorczyca, and M. R. Melamed. 1999. Laser-scanning cytometry: a new instrumentation with many applications. *Exp. Cell Res.* 249:1.
12. van Kooyk, Y., P. van de Wiel-van Kemenade, P. Weder, T. W. Kuijpers, and C. G. Figdor. 1989. Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature* 342:811.
13. Dustin, M. L., and T. A. Springer. 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341:619.
14. Cooper, J. A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105:1473.
15. Coue, M., S. L. Brenner, I. Spector, and E. D. Korn. 1987. Inhibition of actin polymerization by latrunculin A. *FEBS Lett.* 213:316.
16. Bubb, M. R., A. M. Senderowicz, E. A. Sausville, K. L. Duncan, and E. D. Korn. 1994. Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* 269:14869.
17. van Kooyk, Y., and C. G. Figdor. 2000. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr. Opin. Cell Biol.* 12:542.
18. Phatak, P. D., C. H. Packman, and M. A. Lichtman. 1988. Protein kinase C modulates actin conformation in human T lymphocytes. *J. Immunol.* 141:2929.
19. Katanaev, V. L., and M. P. Wymann. 1998. Microquantification of cellular and in vitro F-actin by rhodamine phalloidin fluorescence enhancement. *Anal. Biochem.* 264:185.
20. Smilenov, L. B., A. Mikhailov, R. J. Pelham, E. E. Marcantonio, and G. G. Gundersen. 1999. Focal adhesion motility revealed in stationary fibroblasts. *Science* 286:1172.
21. Yednock, T. A., C. Cannon, C. Vandevvert, E. G. Goldbach, G. Shaw, D. K. Ellis, C. Liaw, L. C. Fritz, and L. I. Tanner. 1995.  $\alpha_4\beta_1$  integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. *J. Biol. Chem.* 270:28740.
22. Reilly, P. L., J. R. Woska, Jr., D. D. Jeanfavre, E. McNally, R. Rothlein, and B. J. Bormann. 1995. The native structure of intercellular adhesion molecule-1 (ICAM-1) is a dimer: correlation with binding to LFA-1. *J. Immunol.* 155:529.
23. Miller, J., R. Knorr, M. Ferrone, R. Houdei, C. P. Carron, and M. L. Dustin. 1995. Intercellular adhesion molecule-1 dimerization and its consequences for adhesion mediated by lymphocyte function associated-1. *J. Exp. Med.* 182:1231.
24. Wojciak-Stothard, B., L. Williams, and A. J. Ridley. 1999. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *J. Cell Biol.* 145:1293.
25. Kanner, S. B., L. S. Grosmaire, J. A. Ledbetter, and N. K. Damle. 1993.  $\beta_2$ -integrin LFA-1 signaling through phospholipase C- $\gamma$ 1 activation. *Proc. Natl. Acad. Sci. USA* 90:7099.
26. Rodriguez-Fernandez, J. L., M. Gomez, A. Luque, N. Hogg, F. Sanchez-Madrid, and C. Cabanas. 1999. The interaction of activated integrin lymphocyte function-associated antigen 1 with ligand intercellular adhesion molecule 1 induces activation and redistribution of focal adhesion kinase and proline-rich tyrosine kinase 2 in T lymphocytes. *Mol. Biol. Cell* 10:1891.
27. Volkov, Y., A. Long, S. McGrath, D. Ni Eidhin, and D. Kelleher. 2001. Crucial importance of PKC- $\beta$ (I) in LFA-1-mediated locomotion of activated T cells. *Nat. Immunol.* 2:508.
28. Constantin, G., M. Majeed, C. Giagulli, L. Piccio, J. Y. Kim, E. C. Butcher, and C. Laudanna. 2000. Chemokines trigger immediate  $\beta_2$  integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13:759.
29. Weiner, O. D., G. Servant, M. D. Welch, T. J. Mitchison, J. W. Sedat, and H. R. Bourne. 1999. Spatial control of actin polymerization during neutrophil chemotaxis. *Nat. Cell Biol.* 1:75.
30. Parsey, M. V., and G. K. Lewis. 1993. Actin polymerization and pseudopod reorganization accompany anti-CD3-induced growth arrest in Jurkat T cells. *J. Immunol.* 151:1881.