

# The leukocyte integrin LFA-1 reconstituted by cDNA transfection in a nonhematopoietic cell line is functionally active and not transiently regulated

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The functional activity of lymphocyte function-associated antigen 1 (LFA-1) on leukocytes can be regulated by T-cell receptor (TCR) stimulation and pharmacologic agents. It was of interest to determine if functionally active LFA-1 could be reconstituted on a nonhematopoietic, LFA-1-negative cell line. We report the expression of LFA-1 and diethylaminoethyl (DEAE) Mac-1  $\alpha\beta$  heterodimers on the cell surface of a fibroblastoid cell line, COS, by DEAE dextran cotransfection of the  $\alpha$  and  $\beta$  subunit cDNAs. Immunoprecipitation studies demonstrated that the  $\alpha$  and  $\beta$  subunits were associated in heterodimers. The  $\alpha$  or  $\beta$  subunit was expressed at lower levels after transfection with the  $\alpha$  or  $\beta$  subunit cDNA alone. Cotransfection of the  $\alpha$  and  $\beta$  subunit cDNAs, but not transfection of  $\alpha$  or  $\beta$  alone, was sufficient to reconstitute intercellular adhesion molecule-1 (ICAM-1) binding activity. Consistent with this observation, LFA-1 on the fibroblastoid cells possesses the activation epitope defined by the L16 monoclonal antibody (mAb). This epitope marks the conversion of LFA-1 from the low to high avidity state on peripheral blood T lymphocytes (PBLs) and is constitutively present on activated cell lines. In contrast to LFA-1 on leukocytes, the functional activity of LFA-1 on fibroblastoid cells was not influenced by phorbol ester treatment. Furthermore, the use of agents that interfere with intracellular signaling, a protein kinase C inhibitor, cAMP analogue, or the combination of a phosphodiesterase inhibitor and adenylyl cyclase activator, did not affect the binding of COS cells expressing LFA-1 to purified ICAM-1.

## Introduction

Lymphocyte function-associated antigen 1 (LFA-1) and the other leukocyte integrins, Mac-

1 and p150,95, are integral to leukocyte cellular interactions during inflammation and the immune response. LFA-1, expressed on virtually all leukocytes, is involved in a variety of immune phenomena including leukocyte-endothelial cell interaction, cytolytic T lymphocyte (CTL)-mediated killing, and antibody-dependent killing by granulocytes and monocytes (reviewed in Kishimoto *et al.*, 1989). LFA-1 has two counter-receptors, intercellular adhesion molecule-1 (Rothlein *et al.*, 1986; Marlin and Springer, 1987) and -2 (Staunton *et al.*, 1989) (ICAM-1 and -2), which are both members of the immunoglobulin superfamily. Mac-1 and p150,95 are expressed primarily on myeloid cells. Both Mac-1 and p150,95 may be involved in iC3b binding (Beller *et al.*, 1982; Myones *et al.*, 1988) and myeloid cell-endothelial cell interaction (Anderson and Springer, 1987; Smith *et al.*, 1989b).

The three leukocyte integrins are  $\alpha\beta$  heterodimers having distinct  $\alpha$  subunits (LFA-1  $\alpha$  or CD11a,  $M_r = 180\,000$ ; Mac-1  $\alpha$  or CD11b,  $M_r = 170\,000$ ; and p150,95  $\alpha$  or CD11c,  $M_r = 150\,000$ ) and a common  $\beta$  subunit (CD18,  $M_r = 95\,000$ ) (Kurzinger and Springer, 1982; Sanchez-Madrid *et al.*, 1983; Kishimoto *et al.*, 1989). These three molecules constitute a subfamily of the integrin family. The three leukocyte integrin  $\alpha$  subunits are more homologous to each other than other integrin receptors and possess an inserted or I domain of 200 amino acids, which is not found in other integrin  $\alpha$  subunits, with the exception of very late activation antigen (VLA-2) (Larson *et al.*, 1989).

A group of patients with a genetic defect in the  $\beta$  subunit resulting in the loss of surface expression of all three leukocyte integrins has demonstrated the significance of the leukocyte integrins in vivo (Anderson and Springer, 1987). Patients with this disease, known as leukocyte adhesion deficiency (LAD), lack pus formation and have recurrent, life-threatening bacterial infections.

Intracellular signals influence the functional activity of LFA-1. For instance, lymphoid cell lines expressing LFA-1 and ICAM-1 do not or only mildly spontaneously aggregate in culture.

Treatment with phorbol esters induces a rapid, sustainable aggregation of these cells, which is dependent on LFA-1 (Patarroyo *et al.*, 1985; Rothlein and Springer, 1986) and ICAM (Rothlein *et al.*, 1986) but is not accompanied by a change in the cell surface density of LFA-1 or ICAM-1 (Rothlein *et al.*, 1986). Treatment of cells with phorbol esters converts LFA-1 but not ICAM-1 from a low to high avidity state, as shown by binding to purified ICAM-1 and LFA-1, respectively (Dustin and Springer, 1989). LFA-1 avidity is also regulated by crosslinking the T-cell receptor (TCR) (Dustin and Springer, 1989). In contrast to the sustained LFA-1 avidity increase produced by phorbol ester, the avidity increase stimulated by TCR crosslinking is transient. Stimulation of LFA-1 avidity through the TCR is inhibitable with cyclic adenosine monophosphate (cAMP) analogues. It was of interest to know if structurally intact LFA-1  $\alpha\beta$  heterodimers could be reconstituted by cotransfecting  $\alpha$  and  $\beta$  subunit cDNAs and if expression of these two subunits alone was sufficient for ligand binding activity and for its regulatability.

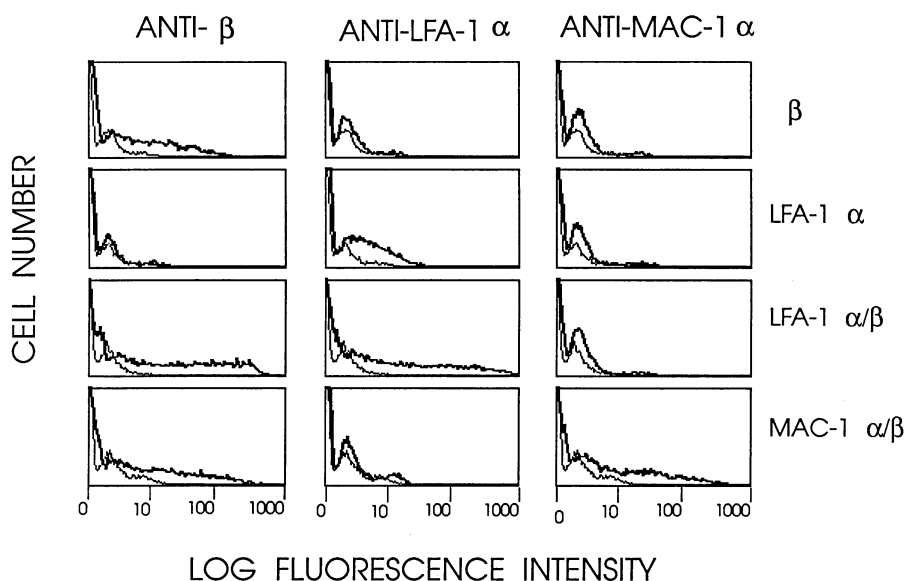
## Results

### Expression of LFA-1 on nonhematopoietic cells

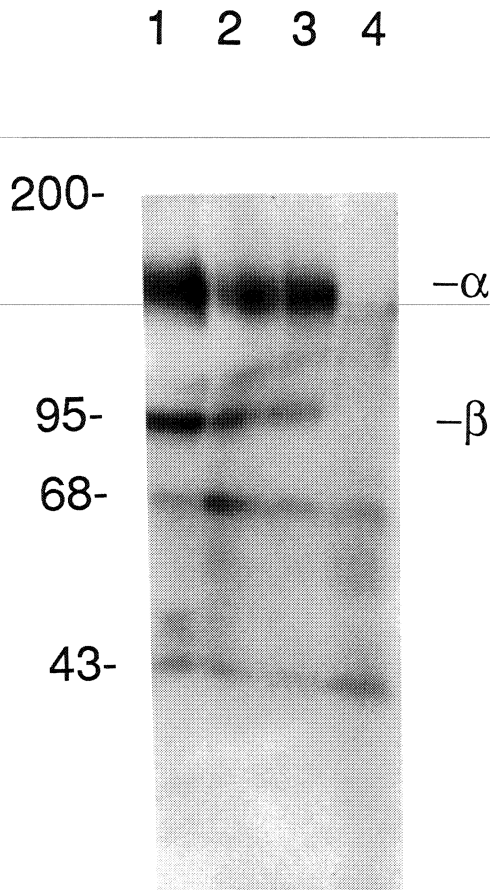
The COS fibroblastoid cell line transfected with either the LFA-1 or Mac-1  $\alpha$  subunit cDNA,  $\beta$  subunit cDNA, or both  $\alpha$  and  $\beta$  cDNA were subjected to immunofluorescent staining and flow cytometry (Fig. 1). LFA-1 and Mac-1  $\alpha\beta$  heterodimers were expressed on the cell surface of 52 and 43% of COS cells, respectively, after

cotransfection of the  $\alpha$  and  $\beta$  subunit cDNAs. LFA-1 and/or Mac-1  $\alpha\beta$  heterodimers on COS cells were detected by 33 of a panel of 34 monoclonal antibodies (mAbs) specific for the appropriate  $\alpha$  subunit or the shared  $\beta$  subunit (Larson *et al.*, 1990). The  $\alpha$  and  $\beta$  subunits were detected on a lower number of cells (32% and 38%, respectively) after transfecting the  $\alpha$  or  $\beta$  subunit cDNA alone. Only a subset of mAb to the  $\beta$  subunit are reactive with the  $\beta$  subunit expressed alone in COS cells (Larson *et al.*, 1990). Comparison of the modal specific linear fluorescence intensity of the transfected cell populations shows that, when  $\alpha$  and  $\beta$  cDNA are cotransfected, LFA-1 is expressed 4-fold and 20-fold better than the respective  $\beta$  or  $\alpha$  subunit alone.

COS cells cotransfected with the  $\alpha$  and  $\beta$  cDNAs were examined by immunoprecipitation. Antibodies directed to the  $\alpha$  or  $\beta$  subunits immunoprecipitated both subunits, indicating that both subunits were associated on the cell surface (Fig. 2, lanes 2 and 3). Each subunit had the same apparent molecular weight as material immunoprecipitated from SKW3 cells, a human T-lymphoma cell line (Fig. 2, lane 1). Furthermore, preclearing of the lysate with an anti- $\beta$  subunit antibody removed material immunoprecipitated with the  $\alpha$  subunit mAb (Fig. 2, lane 4). We have been able to immunoprecipitate the  $\beta$  subunit from COS cells transfected with  $\beta$  cDNA alone; no coassociated  $\alpha$  subunit has yet been detected (not shown). This does not rule out association with an integrin  $\alpha$  subunit indigenous to COS cells, because this may be difficult to detect for technical reasons.



**Figure 1.** Immunofluorescence flow cytometry of COS cells after transfection with  $\alpha$  and  $\beta$  subunit cDNA alone or together. The mAb used to detect surface protein is labeled on top (anti- $\beta$ , TS1/18; anti-LFA-1  $\alpha$ , TS1/22; anti-Mac-1  $\alpha$ , LM 2/1) and the cDNAs used for transfection are listed on the right.



**Figure 2. SDS-PAGE of <sup>125</sup>I-labeled material.** Lane 1: SKW3 lysate immunoprecipitated with anti- $\alpha$  subunit mAb (TS1/22). Lanes 2 and 3: COS cells cotransfected with LFA-1  $\alpha$  and  $\beta$  subunit cDNAs and immunoprecipitated with anti- $\alpha$  subunit and anti- $\beta$  subunit mAb (TS1/22 and TS1/18, respectively). Lane 4: LFA-1-bearing COS cell lysate pre-cleared with TS1/18 and immunoprecipitated with TS1/22.

**Functional integrity of expressed LFA-1  $\alpha\beta$  heterodimers**

Transfected COS cells were examined for their ability to bind purified ICAM-1 (Fig. 3). The LFA-1-bearing COS cells bound to purified ICAM-1, and the binding was inhibitable with EDTA and mAb directed against ICAM-1 and the LFA-1  $\alpha$  and  $\beta$  subunits. In contrast, COS cells expressing similar amounts of Mac-1 and mock-transfected COS cells did not interact significantly with ICAM-1. Mac-1 expressed by the transfected COS cells was functionally intact; COS cells cotransfected with Mac-1  $\alpha$  and  $\beta$  subunit cDNAs—but not with LFA-1  $\alpha$  and  $\beta$  subunit cDNAs—bound to erythrocytes sensitized with the iC3b complement fragment (Aguilar, Corbi, and Springer, in preparation).

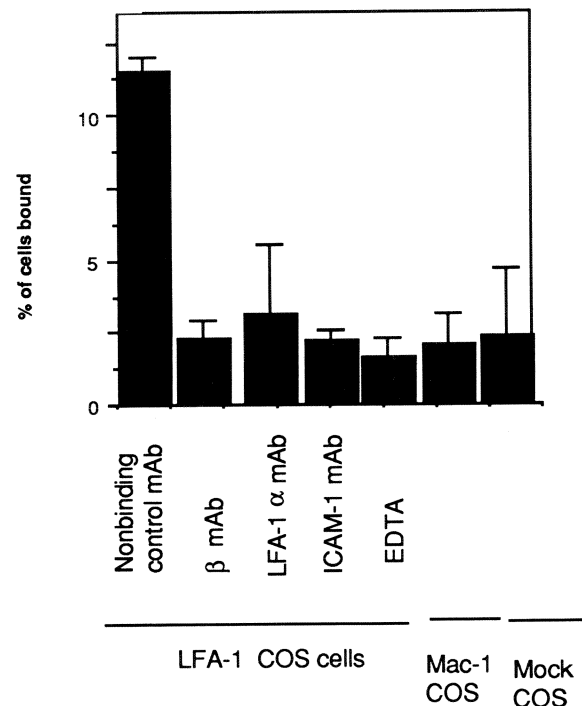
COS cells transfected with the  $\alpha$  or  $\beta$  subunit

cDNA alone or together were examined for their ability to interact with ICAM-1 (Fig. 4). Although COS cells expressing the LFA-1  $\alpha\beta$  complex bound specifically to ICAM-1, COS cells expressing the  $\alpha$  or  $\beta$  subunit alone did not bind significantly above background levels.

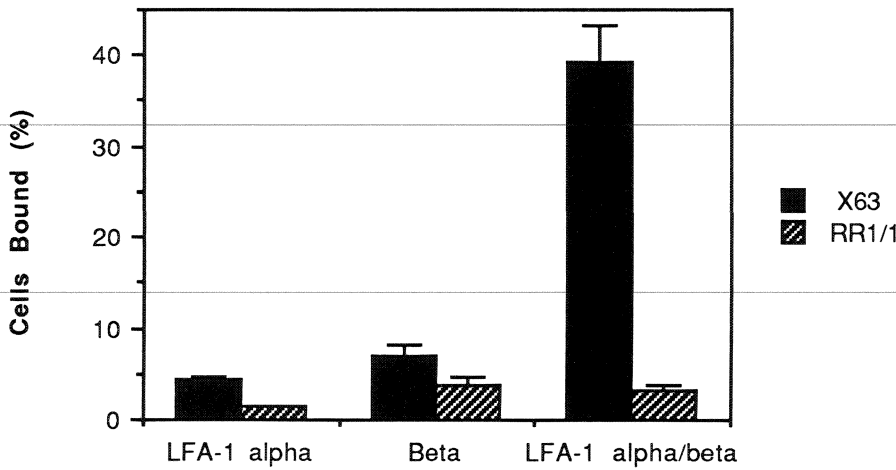
**Regulation of binding by phorbol esters**

COS cells expressing LFA-1 were examined for their ability to bind purified ICAM-1 in the presence or absence of phorbol ester in parallel with resting peripheral blood T lymphocytes (PBLs) and with a B lymphoblastoid cell line, JY (Fig. 5). JY cell and PBL binding to ICAM-1 was stimulated two- to fivefold by phorbol ester. In contrast, COS cells expressing LFA-1 did not demonstrate a significant increase in binding to ICAM-1.

Treatment of COS cells expressing LFA-1 with a cell-permeable cAMP analogue (dibutyryl cAMP), the combination of a phosphodiesterase inhibitor and adenylyl cyclase stimulator (isobutyl methyl xanthine [IBMX] and forskolin, respectively), a protein kinase C inhibitor (staurosporine), or staurosporine and phorbol 12-myristate



**Figure 3. Binding of COS cells expressing LFA-1 and Mac-1 to purified ICAM-1 adsorbed to plastic.** Cells were 44% and 43% positive for LFA-1 and Mac-1, respectively. Binding was in the presence of the indicated additions. MAbs were TS1/22 (anti-LFA-1  $\alpha$  subunit), TS1/18 (anti- $\beta$  subunit), RR1/1 (anti-ICAM-1), and X63 (nonbinding control).



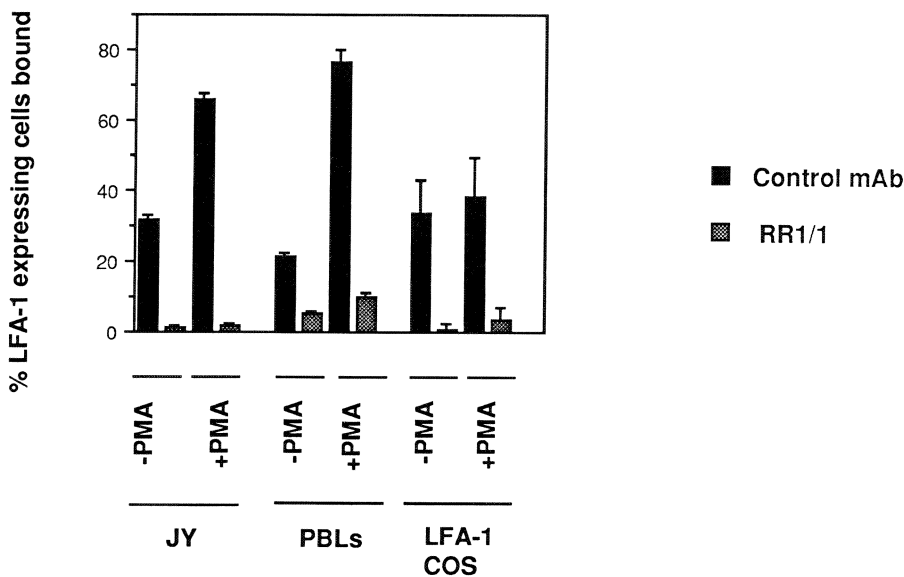
**Figure 4. Binding of COS cells transfected with LFA-1  $\alpha$  and  $\beta$  cDNAs together or alone.** Cells cotransfected with the  $\alpha$  and  $\beta$  subunit cDNA or with each subunit cDNA alone were 55% ( $\alpha\beta$ ), 25% ( $\alpha$ ), and 40% ( $\beta$ ) positive, respectively. Solid and striped bars show binding to ICAM-1 in the presence of control X63 mAb or ICAM-1 RR 1/1 mAb, respectively.

13-acetate (PMA) was also examined (Fig. 6). These agents were used at concentrations previously tested for inhibition of the increased adhesiveness of LFA-1 that can be stimulated by TCR crosslinking. Dibutyryl cAMP or IBMX together with forskolin inhibit the TCR-stimulated, but not the PMA-stimulated increase in LFA-1 adhesiveness. The PMA-stimulated increase is inhibited by staurosporine (Dustin and Springer, 1989). None of the agents or combinations of agents used had an effect on the binding of COS cells expressing LFA-1 to purified ICAM-1 (Fig. 6A). Butyrate, a control for effects of butyrate released from dibutyryl cAMP degradation, and dimethyl sulfoxide (DMSO) buffer control also did not affect COS cell binding. In contrast, the presence of staurosporine partially inhibited phorbol ester-induced binding of JY cells to

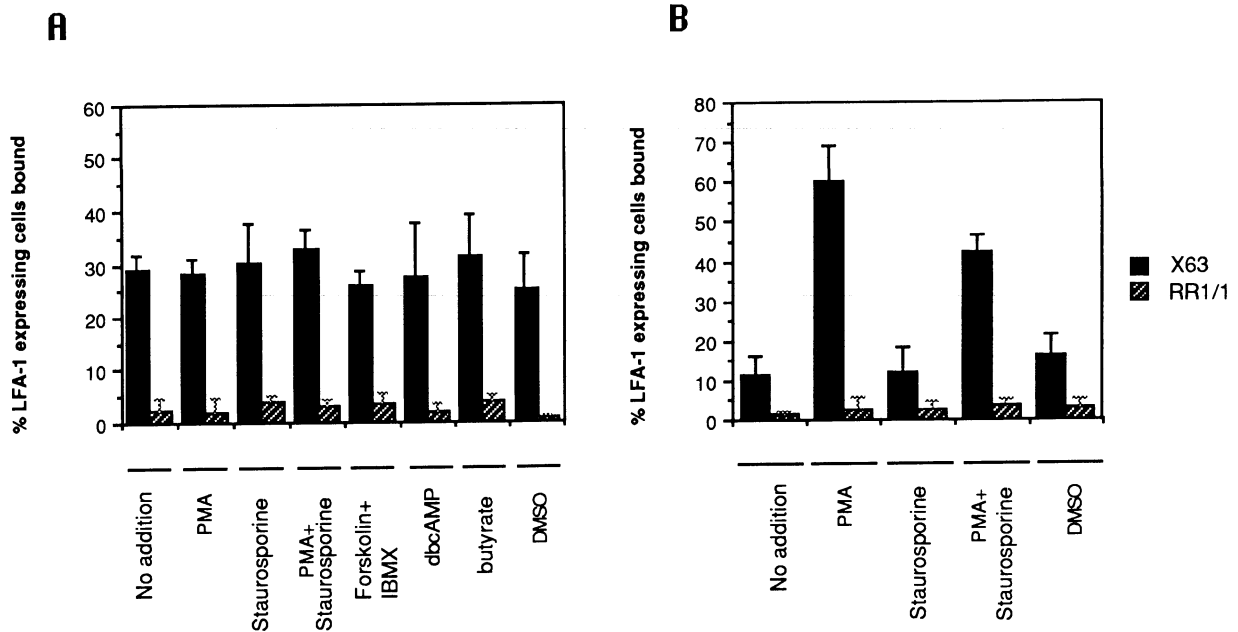
ICAM-1 (Fig. 6B). The specificity of these interactions in all cases was demonstrated by inhibition with an anti-ICAM-1 mAb.

**Expression of an LFA-1 activation epitope**

The mAb L16 recognizes the  $\alpha$  subunit of LFA-1 and induces cell-cell aggregation that can be inhibited by mAb to other LFA-1 epitopes (Keizer *et al.*, 1988). The L16 epitope is expressed only weakly on resting PBLs and induced by culturing for several days in PMA or interleukin-2 (IL-2). This increase in the L16 epitope correlates with increased functional activity of LFA-1 on CTLs (C. Figdor, personal communication). The L16 mAb recognized LFA-1 expressed on COS cells after cotransfection of  $\alpha$  and  $\beta$  subunit cDNAs (Fig. 7A). L16 epitope



**Figure 5. Phorbol ester stimulation of binding to ICAM-1.** Solid bars indicate binding of each cell type in presence of control TS2/4 mAb (anti-LFA-1  $\alpha$  nonblocking mAb), whereas hatched bars demonstrate binding in presence of anti-ICAM-1 mAb RR1/1. Transfected COS cells were 49% LFA-1 positive. The percent LFA-1 expressing cells bound was determined by the following equation:  $(\text{cpm bound} / [\text{input cpm} \times \text{fraction of LFA-1 expressing cells}]) \times 100$ .



**Figure 6.** Binding of COS cells expressing LFA-1 (A) and JY cells (B) to ICAM-1 in the presence of dibutyl cAMP (dbcAMP), staurosporine alone, staurosporine and PMA, forskolin and IBMX, butyrate, or DMSO. LFA-1 transfected COS cells were 54% positive. Solid and striped bars show the binding to ICAM-1 in the presence of control X63 mAb or ICAM-1 RR1/1 mAb, respectively.

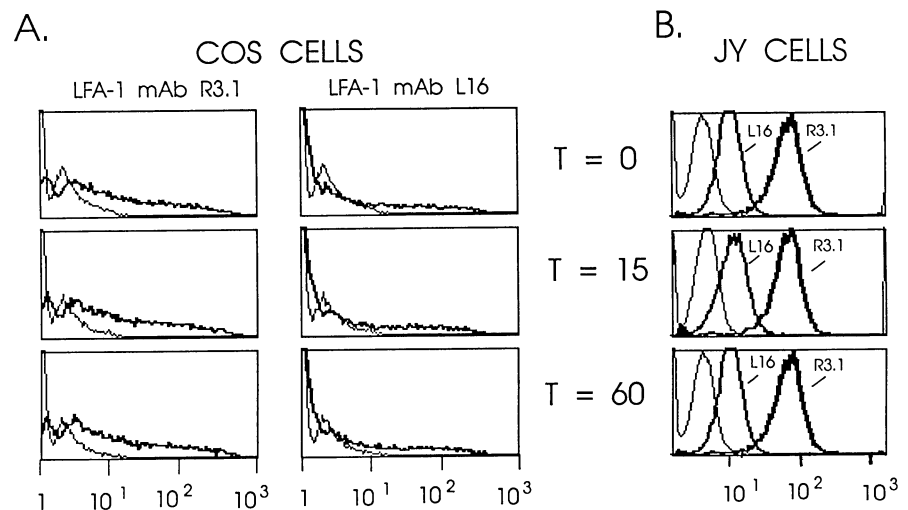
is expressed at a lower density than the epitope defined by other LFA-1 mAb such as the isotype-matched mAb R3.1. Expression of the L16 epitope on transfected COS cells is not influenced by stimulation with phorbol esters for 15 or 60 min (Fig. 7A).

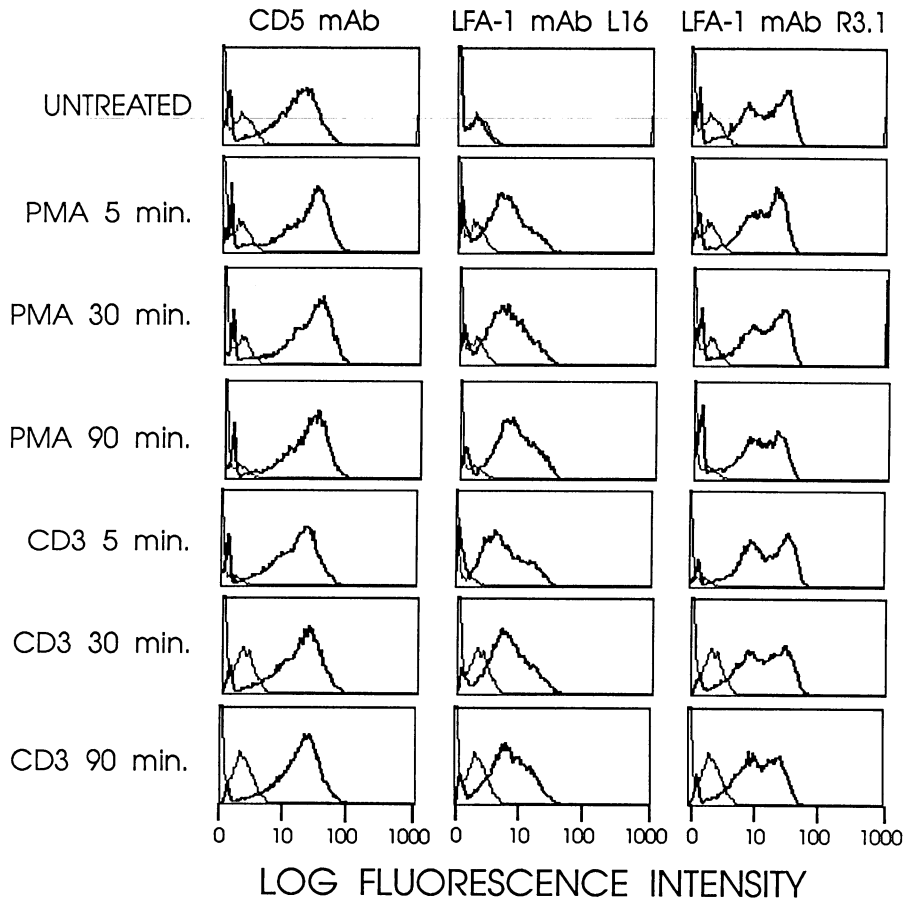
The JY lymphoblastoid B cell line also expresses the L16 epitope at lower density than epitopes defined by other LFA-1 mAb (Fig. 7B).

PMA stimulation does not enhance expression of the L16 epitope on JY cells (Fig. 7B), although it does induce LFA-1-dependent homotypic adhesion of these cells.

In contrast to transfected COS cells and lymphoblastoid cells, the L16 epitope is absent from LFA-1 on resting PBLs (Fig. 8). PBLs were stimulated with phorbol ester or by crosslinking the TCR with a mAb to the noncovalently associated

**Figure 7.** Immunofluorescent staining of LFA-1 epitopes on transfected COS cells (A) and JY cells (B) after phorbol ester stimulation. The mAbs R3.1 and L16 stained  $58 \pm 4.4\%$  (SLFI =  $11 \pm 0.4$ ) and  $23 \pm 3.0\%$  (SLFI =  $4 \pm 0.2$ ) of transfected COS cells, respectively. The SLFI of JY cells stained with R3.1 and L16 were  $82 \pm 4.8$  and  $9.6 \pm 0.7$ , respectively. The isotype-matched (IgG2a) LFA-1 mAb R3.1 and L16, and the time in minutes after PMA stimulation are indicated. The nonbinding negative control (X63 light line) is superimposed. The X and Y axis represent the log fluorescence intensity and cell number, respectively.





**Figure 8.** Induction of L16 epitope on peripheral blood T cells by phorbol ester treatment or TCR crosslinking. The mAb used for immunofluorescence is indicated above, and the nature and duration of stimulation is indicated on the left. The X and Y axes represent the log fluorescence intensity and cell number, respectively.

invariant CD3 subunits and a goat anti-mouse IgG polyclonal second antibody. After either stimulus, the L16 epitope was induced after 5 min and then underwent no further change at 15, 30, 60, and 90 minutes (Fig. 8 and not shown). Although some of the fluorescence-activated cell sorter (FACS) profiles suggest a bimodal distribution of L16<sup>+</sup> cells, two populations are not clearly distinguishable. MAb to LFA-1 epitopes that are constitutively present recognize two populations of LFA-1<sup>+</sup> cells. These high and low LFA-1 expressing T-cell populations have been previously described (Kürzinger *et al.*, 1981; Sanders *et al.*, 1988). The kinetics of induction of the L16 epitope and conversion of LFA-1 from a low to a high avidity state (Dustin and Springer, 1989) are similar, both occurring within 5 min of phorbol ester treatment or CD3-cross-linking.

### Discussion

We have shown that structurally and functionally intact LFA-1  $\alpha\beta$  heterodimers can be expressed after cotransfection of a nonhematopoietic cell

line with  $\alpha$  and  $\beta$  subunit cDNAs. Transfection with cDNA for the  $\alpha$  or  $\beta$  subunits of LFA-1 alone results in expression but at levels 4- to 20-fold less than with cotransfection. COS cells transfected with only the LFA-1  $\alpha$  or  $\beta$  subunit lack the ability to bind ICAM-1. LAD patient cells genetically deficient in the  $\beta$  subunit do not express any  $\alpha$  subunit on the cell surface (Anderson and Springer, 1987). The COS cell expression system greatly overproduces the  $\alpha$  and  $\beta$  subunits intracellularly. This overexpression may either drive the individually transfected  $\alpha$  or  $\beta$  subunits to be expressed alone or may drive association with an inappropriate integrin subunit, allowing heterologous  $\alpha\beta$  heterodimers to come to the cell surface. COS cells abundantly express  $\beta_1$  (CD29) integrins.

Cotransfection of the  $\alpha$  and  $\beta$  subunit cDNAs was sufficient to reconstitute LFA-1 functional activity, and transfection with individual subunits was not sufficient. Binding to ICAM-1 by transfected cells was not enhanced by phorbol ester stimulation. This observation suggests that LFA-1 expressed on fibroblastoid cells is

not functionally regulated. In contrast, PMA stimulates adhesion of PBLs and B lymphoblastoid cell to ICAM-1, as previously demonstrated (Dustin and Springer, 1989). Two possibilities can exist to explain this observation. First, expression of LFA-1 on the cell surface is not sufficient for functional activity, and a positive intracellular signal converts LFA-1 to a functional form. This signal would be constitutively present in COS cells but regulated in leukocytes. Alternatively, LFA-1 expression is sufficient for ICAM-1 binding activity and a regulatable negative signal controls activity. In this case the negative signal would not be present in COS cells but regulated in leukocytes.

PMA-stimulated increase in LFA-1 avidity on resting T cells (Dustin and Springer, 1989) is completely inhibited by staurosporine. We found that JY cell binding after PMA stimulation to ICAM-1 is partially inhibited by staurosporine. In contrast, binding of COS cells expressing LFA-1 and baseline binding of JY cells to ICAM-1 were not inhibited by the use of a protein kinase C inhibitor, cAMP analogue, or the combination of an adenylyl cyclase activator and phosphodiesterase inhibitor.

Binding to ICAM-1 of COS cells cotransfected with LFA-1 but not Mac-1  $\alpha$  and  $\beta$  cDNAs was apparent in our assay system, which allows adhesion under static conditions followed by stringent washing by aspiration, which creates a high shear force. An interaction between Mac-1 and ICAM-1 is suggested, however, in studies using washing by  $1 \times g$  inversion to separate Mac-1<sup>+</sup> neutrophils from ICAM-1-containing planar membranes (Smith *et al.*, 1989a). The more stringent wash conditions in our assay may not allow a lower affinity Mac-1/ICAM-1 interaction to be detected. Indeed, independent studies confirm that Mac-1 is a receptor for ICAM-1, however, the interaction is weaker, more temperature sensitive, and less shear resistant than for the interaction of LFA-1 with ICAM-1 (M.L. Diamond and T.A. Springer, unpublished data).

We examined the expression of an LFA-1 activation epitope, L16, on COS cells and examined the kinetics of its appearance after stimulation of peripheral blood T lymphocytes to allow comparison to the kinetics of induction of LFA-1 adhesiveness (Dustin and Springer, 1989). The L16 epitope appears after stimulation of resting T lymphocytes with kinetics similar to the conversion of LFA-1 from the low to high avidity state; however, the L16 epitope remains expressed after 30 min and thus, in contrast to the high avidity state, is not transient.

Although phorbol esters induce adhesiveness of LFA-1 on JY cells (Dustin and Springer, 1989), this did not alter the L16 epitope on these cells. If the L16 epitope and the avidity state are related to conformational changes in the LFA-1 molecule, our findings, and those of Figdor and coworkers (personal communication) suggest at least three different conformational states: low avidity, L16<sup>-</sup>; high avidity, L16<sup>+</sup>; and low avidity, L16<sup>+</sup>. Thus, although it is of interest that LFA-1 expressed in COS cells bears the L16 epitope, this cannot be correlated with the ability of LFA-1 on these cells to bind ICAM-1.

We have shown that LFA-1 molecules expressed on transfected COS cells are intact  $\alpha\beta$  heterodimers that bind ICAM-1. Further studies are now possible to use transfection of mutated LFA-1  $\alpha$  and  $\beta$  subunits to elucidate the ICAM-1 binding region(s) and to determine whether transmembrane or cytoplasmic regions of these subunits are involved in regulating the avidity of LFA-1 for ICAM-1 by interacting with other cellular components.

## Materials and methods

### mAbs

The mAbs used in these experiments include TS1/22, TS2/4 (Sanchez-Madrid *et al.*, 1982), L16 (Keizer *et al.*, 1988), and R3.1 (Smith *et al.*, 1989a) to LFA-1  $\alpha$ ; TS1/18 (Sanchez-Madrid *et al.*, 1982) to  $\beta$ ; LM2/1 (Miller *et al.*, 1986) to Mac-1  $\alpha$ ; RR1/1 (Rothlein *et al.*, 1986) to ICAM-1; Leu-4 (Friedrich *et al.*, 1982) to CD3; Leu-1 (Wang *et al.*, 1980) to CD5; and X63 (Köhler and Milstein, 1975) (nonbinding control).

### Diethylaminoethyl (DEAE)-dextran transfection of COS cells

cDNAs containing the entire coding region of the LFA-1  $\alpha$  and  $\beta$  subunit cDNAs (Larson *et al.*, 1989; Kishimoto *et al.*, 1987) were subcloned into the expression vector CDM8. The subcloning of the  $\beta$  subunit cDNA has been described (Hibbs *et al.*, 1990). A 2.5-kb  $\alpha$  subunit insert in the  $\lambda$ gt10 clone 5L5 (Larson *et al.*, 1989) was removed by complete digestion with *Sph* I and *Eco*RI and inserted in the plasmid vector, Sp65 (Promega, Madison, WI). A 5' 1.8-kb *Eco*RI fragment containing the rest of the LFA-1  $\alpha$  subunit coding sequence was isolated from the  $\lambda$ gt10 clone 3R1 and ligated to the 3' fragment in Sp65 described above. The proper orientation of the 5' insert was determined by complete digestion with *Sal* I. A 4.5-kb fragment that contained the entire coding sequence of the LFA-1  $\alpha$  subunit and the polylinker of Sp65 from *Hind*III to *Eco*RI was isolated by complete digestion with *Hind*III and *Sph* I. This fragment was ligated with *Bst* XI linkers following blunting of the *Hind*III and *Sph* I sites with T4 DNA polymerase, and subcloned into the *Bst* XI site in CDM8 (Seed and Aruffo, 1987). The  $\alpha$  and  $\beta$  subunit cDNAs were cotransfected into COS cells, a monkey kidney fibroblastoid cell line, using DEAE-dextran (Seed and Aruffo, 1987; Larson *et al.*, 1990).

### Immunoprecipitation of cell surface labeled material

COS cells ( $1 \times 10^6$ ) transfected with both LFA-1  $\alpha$  and  $\beta$  subunit cDNA or SKW3 cells, a T-lymphoma cell line, were

labeled with  $^{125}\text{I}$  (Fraker and Speck, 1978) and lysed in 1 ml of TSA (20 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.05% sodium azide), 1.0% Triton X-100. Cell lysates were precleared with 30  $\mu\text{l}$  of a 1:1 slurry of anti-Ig or TS1/22 mAb coupled to Sepharose CL-4B for 4 h at 4°C. Cell lysate ( $2 \times 10^6$  cpm) was incubated for 2 h at 4°C with 30  $\mu\text{l}$  of culture supernatant or 1:100 ascites in phosphate-buffered saline (PBS) in Eppendorf tubes precoated with 1% hemoglobin in TSA. A 1:1 slurry of anti-Ig coupled to Sepharose (Sigma) (15  $\mu\text{l}$ ) was added, and each tube was incubated an additional 2 h at 4°C. Each tube was washed twice with TSA, 1% Triton X-100, 0.1% hemoglobin, once with TSA, 1% Triton X-100, and once with 50 mM Tris-HCl (pH 6.8). Samples were heated to 100°C in sodium dodecyl sulfate sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli and Favre, 1973).

### Indirect immunofluorescence

COS cells harvested with 5 mM EDTA, RPMI 1640 (GIBCO, Grand Island, NY) were washed once with cold PBS (1.4 mM potassium monobasic phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium dibasic phosphate pH 7.3), 1 mM EDTA. The supernatant was removed and the cells were incubated 30 min on ice in 50  $\mu\text{l}$  cold PBS and 50  $\mu\text{l}$  culture supernatant containing the appropriate antibody or a 1:100 dilution of ascites in PBS. Cells were washed twice with PBS and incubated an additional 30 min on ice in 50  $\mu\text{l}$  PBS and 50  $\mu\text{l}$  of a 1:10 dilution of 1 mg/ml solution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse whole Ig polyclonal antibody (Zymed Laboratories, South San Francisco, CA) or FITC-goat anti-mouse IgG2a polyclonal antibody (Calgene, South San Francisco, CA). EDTA was omitted from all solutions when the mAb L16 was used. Cells were washed two additional times with PBS and resuspended in 125  $\mu\text{l}$  PBS, 1% paraformaldehyde. The percent cellular expression and specific linear fluorescence intensity (SLFI) were determined (Kürzinger *et al.*, 1981) on an EPICS V (Coulter, Hialeah, FL).

### Isolation of PBLs

Resting PBLs were freshly isolated from whole blood by dextran sedimentation, plastic adherence, and nylon wool filtration. Cell populations were typically 75–95% T cells.

### Cell binding to purified ICAM-1

ICAM-1 was purified by affinity chromatography and adsorbed to plastic in 96-well plates (Titertek Flow Laboratory, McLean, VA) as previously described (Dustin and Springer, 1989). Antibodies (50  $\mu\text{l}$ ) in culture supernatant or as 1:200 ascites diluted in culture medium were added to each well along with  $^{51}\text{Cr}$ -labeled COS cells ( $4 \times 10^4$ ), the B lymphoblastoid cell line JY ( $8 \times 10^4$ ), or PBLs ( $2 \times 10^5$ ) in culture medium. For phorbol ester stimulation, cells were treated with PMA (Sigma) for varying time periods at a final concentration of 50 ng/ml, 37°C. Forskolin (25  $\mu\text{M}$ , 0.5% DMSO)(Calbiochem, La Jolla, CA), dibutyryl cAMP (2 mM) (Calbiochem), IBMX (0.5 mM, 0.5% DMSO (Sigma), and staurosporine (5  $\mu\text{g}/\text{ml}$ , 0.5% DMSO)(Boehringer Mannheim, Mannheim, West Germany) were added at concentrations previously reported to inhibit TCR signaling (Dustin and Springer, 1989). Butyrate (4mM) and DMSO (0.5%) were used as additional controls. CD3 crosslinking was done as previously described (Dustin and Springer, 1989). The cells were incubated for 15 min or as indicated at room temperature, redispersed by pipetting, spun at  $37 \times g$  (clinical centrifuge, Beckman, Wakefield, MA) to bring the cells in contact

with the ICAM-1, and incubated at 37°C for 30 min. Each well was then washed four to six times with room temperature culture medium, the wash solution being aspirated with a 21-ga needle. The cells were lysed with 0.1 N sodium hydroxide and the number of counts per minute bound was measured on a gamma counter (Beckman).

### Acknowledgments

We thank Mr. Ed Luther for fluorescence flow cytometer operation and Drs. Robert Rothlein and Carl Figdor for donation of the R3.1 and NKI-L16 mAb. This work was supported by NIH grant CA31798. Dr. M.L. Hibbs was supported by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

Received: December 7, 1989.

Revised and accepted: February 20, 1990.

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