# Extracellular Ca$^{2+}$ Modulates Leukocyte Function-associated Antigen-1 Cell Surface Distribution on T Lymphocytes and Consequently Affects Cell Adhesion

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*Abstract.* Transition of leukocyte function-associated antigen-1 (LFA-1), from an inactive into an activate state depends on the presence of extracellular Mg$^{2+}$ and/or Ca$^{2+}$ ions. Although Mg$^{2+}$ is directly involved in ligand binding, the role of Ca$^{2+}$ in LFA-1 mediated adhesion remained obscure. We now demonstrate that binding of Ca$^{2+}$, but not Mg$^{2+}$, directly correlates with clustering of LFA-1 molecules at the cell surface of T cells, thereby facilitating LFA-1-ligand interaction. Using a reporter antibody (NKI-L16) that recognizes a Ca$^{2+}$-dependent epitope on LFA-1, we found that Ca$^{2+}$ can be bound by LFA-1 with different strength. We noticed that weak binding of Ca$^{2+}$ is associated with a dispersed LFA-1 surface distribution on T cells and with non-responsiveness of these cells to stimuli known to activate LFA-1. In contrast, stable binding of Ca$^{2+}$ by LFA-1 correlates with a patch-like surface distribution and vivid ligand binding after activation of LFA-1. Mg$^{2+}$-dependent ligand binding does not affect binding of Ca$^{2+}$ by LFA-1 as measured by NKI-L16 expression, suggesting that Mg$^{2+}$ binds to a distinct site, and that both cations are important to mediate adhesion. Only Sr$^{2+}$ ions can replace Ca$^{2+}$ to express the L16 epitope, and to induce clustering of LFA-1 at the cell surface.

We conclude that Ca$^{2+}$ is involved in avidity regulation of LFA-1 by clustering of LFA-1 molecules at the cell surface, whereas Mg$^{2+}$ is important in regulation of the affinity of LFA-1 for its ligands.

T$_{HE}$ leukocyte function-associated antigen-1 (LFA-1)[1] is a cell adhesion molecule, which mediates different adhesion processes occurring between leukocytes (Springer et al., 1982, 1987; Martz, 1987). LFA-1 (CD11a/CD18) is a member of the $\beta$2 family of the integrins (Hynes, 1987). It consists of an $\alpha$ chain and a $\beta$ chain, which are non-covalently linked (Keizer et al., 1985). Cell adhesion mediated by LFA-1 is established by binding of LFA-1 to its ligands on the opposing cell. Ligands for LFA-1 are intercellular adhesion molecule-1 (ICAM-1), -2 and -3 (Marlin et al., 1987; Staunton et al., 1989; de Fougerolles et al., 1991, 1992).

LFA-1/ICAM-1-mediated adhesion can be regulated by changes in the affinity of LFA-1 for its ligand ICAM-1. Triggering of cell surface receptors CD2, CD3, or MHC class II can induce such an affinity change in LFA-1, thereby inducing cell-cell adhesion (Dustin et al., 1989; van Kooyk et al., 1989; Mourad et al., 1990). Also activation of PKC by PMA can switch the inactive LFA-1 molecule into an active conformation (Rothlein et al., 1986; Patarroyo et al., 1985; Chatila et al., 1989; Buyon et al., 1990). These data point out that the LFA-1 molecule can become activated by intracellular signals (Dustin et al., 1989; van Kooyk et al., 1989), and indicates that at least two forms of LFA-1 exist: "inactive LFA-1" and "active LFA-1." We previously described that binding of an unique antibody (NKI-L16), directed against the $\alpha$ chain of LFA-1, can stimulate LFA-1-mediated adhesion (Keizer et al., 1988) by inducing a conformational change, without the generation of intracellular signals (van Kooyk et al., 1991). We have demonstrated that LFA-1 can only become activated when it expresses the L16 epitope ("potentially active LFA-1") (Figdor et al., 1990). Since NKI-L16 antibodies recognize a Ca$^{2+}$-dependent epitope located on the $\alpha$ chain of LFA-1, Ca$^{2+}$ ions may be important in the transition of one form into the other (van Kooyk et al., 1991).

The critical role of extracellular divalent cations (Mg$^{2+}$ and Ca$^{2+}$) in LFA-1/ligand interaction suggests that structural alterations necessary for ligand recognition may occur as a result of divalent cation binding (Martz, 1980; Rothlein et al., 1986; Makgoba et al., 1988; Marlin et al., 1987). The

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1. *Abbreviations used in this paper:* ICAM, intercellular adhesion molecule; IPB, immunoprecipitation buffer; LFA, leukocyte function-associated antigen; PBL, peripheral blood lymphocytes; RT, room temperature.

three putative cation binding motifs (EF-hand like domains) in the $\alpha$ subunit of the LFA-1 family (Larson et al., 1989), and the presence of clustered oxygenated residues in the $\beta2$ chain, have both been proposed to provide divalent cation binding (Loftus et al., 1990; Dransfield, 1991; Ginsberg et al., 1991). The finding that the binding of $Mn^{2+}$ to LFA-1 can augment LFA-1/ligand interaction, as well as other integrin mediated interactions (Gailit et al., 1988; Kirchhofer et al., 1990; Elices et al., 1991; Sonnenberg et al., 1988; Altieri, 1991), indicates that binding of certain divalent cations can induce conformational changes in LFA-1 (Dransfield et al., 1992), which may alter the affinity of LFA-1 for its ligand. The importance of $Mg^{2+}$ ions in LFA-1/ligand interaction has been illustrated by a $Mg^{2+}$-dependent LFA-1 antibody (mAb 24) (Dransfield et al., 1989; 1992). In these studies, the high affinity change of LFA-1 induced by ligand binding, corresponded with expression of the 24 epitope, indicating that $Mg^{2+}$ binding induces a high affinity change in LFA-1.

By using the NKI-L16 antibody as a reporter for $Ca^{2+}$ occupancy of the LFA-1 receptor, we investigated on different T cells the role and function of $Ca^{2+}$ in LFA-1 distribution and adhesion, and the effect of other divalent cations herein.

## Materials and Methods

### mAbs and Chemicals

The mAbs SPV-L7 (IgG1), NKI-L15 (IgG2A), and NKI-L16 (IgG2A) all reactive with the $\alpha$ chain of LFA-1 (CD11a) were raised as described previously (Keizer et al., 1985, 1988). mAb F10.2 was directed against ICAM-1 (Bloemen et al., 1992). The reagents used were; PMA (phorbol 12-myristate 13-acetate, 50 ng/ml; Sigma Chemical Co., St. Louis, MO); Chelex 100 micro spheres (Biorad, 1% wt/vol), to deplete solutions for the presence of divalent cations by rotary mixing for 4 h at 4°C; EDTA or EGTA (Fluka Chemie AG, Buchs, Switzerland; 5 mM) to remove cell bound divalent cations by incubation of cells for 15 min at 37°C, viability of the cells was always >95%. Divalent cations (CaCl$_2$, MgCl$_2$, ZnCl$_2$, CoCl$_2$, CuCl$_2$, NiCl$_2$, and SrCl$_2$; Sigma Chemical Co.) were diluted in Hepes/NaCl buffer.

### Cell Lines and Cell Culture

The human T cell clone JS136 (CD3$^+$ CD4$^+$ CD16$^-$) (Borst et al., 1986) used in this study, was cultured as described previously (van Kooyk et al., 1991). T cell line Peer was cultured in Iscove's medium containing 5% FCS. A homogenous population of highly purified resting T lymphocytes was isolated from buffy coats of healthy donors by centrifugal elutriation, as described (van Kooyk et al., 1991), and were cultured in Iscove's medium containing 5% FCS.

### Fluorescence Analysis

Cells were washed twice in Hepes/NaCl buffer (cation free), after which they were incubated (30 min, 4°C) with purified antibody (5 $\mu$/ml) in the presence of appropriate dilution's cations. Cells were washed twice in PBS, containing 1% wt/vol bovine serum albumin (BSA; Sigma Chemical Co.) and 0.01% sodium azide (PBS/BSA/azide) followed by incubation with fluorescein isothiocyanate (FITC)-labeled goat F(ab')$_2$ anti-mouse IgG antibody (Nordic, Tilburg, The Netherlands) for 30 min at 4°C. Cells were washed once, and the relative fluorescence intensity was measured by FACScan analysis (Becton Dickinson, Mountain View, CA). Cells activated with anti-CD3, anti-CD2, or anti-MHC class II antibodies (10 $\mu$g/ml; all IgG1), were stained with NKI-L16 (IgG2A) or NKI-L15 (IgG2A), and expression was determined with isotype specific FITC-labeled goat anti-mouse IgG2A (Boehringer Mannheim, Mannheim, Germany).

### Confocal Microscopy

Cells were preincubated for 15 min with 5 mM EDTA, 5 mM EGTA, or medium at 37°C, followed by labeling with Fab fragments of SPV-L7 antibody (1:100) in the presence of EDTA or EGTA or 3 mM SrCl$_2$, or 3 mM MgCl$_2$ for 30 min at room temperature (RT). Cells were washed with PBS/BSA/azide followed by incubation with FITC-labeled goat F(ab')$_2$ anti-mouse IgG for 30 min at RT. Cells were washed and fixed with 0.5% paraformaldehyde for 30 min at RT. Cells were attached to poly-L-lysine-coated glass, after which fluorescence distribution was determined by Confocal Laser Microscopy at 488 nm with a krypton/argon laser (Bio Rad Laboratories, Richmond, CA). Dot sizes were defined using as digital image analyser a TCL image program of Macintosh. The relative size of distribution of LFA-1 is depicted in a scale of 10 to 3300, in which a scale of 100 corresponds +/− 0.33 $\mu$m.

### Binding Assay to L Cells Transfected with ICAM-1

L cells transfected with ICAM-1 (L-ICAM-1) or mock-transfected control L cells (van Kooyk et al., 1993), were grown to form a monolayer of cells. Na$_2$$^{51}$CrO$_4$-labeled T cells were preincubated for 15 min at 4°C in Hepes/NaCl buffer containing divalent cations (1 mM) in the presence of PMA (50 ng/ml), or with the adhesion inducing antibody NKI-L16 (10 $\mu$g/ml). $^{51}$Cr-labeled T cells were preincubated for 15 min at 4°C in Hepes/NaCl buffer containing divalent cations (1 mM) in the presence of PMA (50 ng/ml), or with the adhesion inducing antibody NKI-L16 (10 $\mu$g/ml). $^{51}$Cr-labeled T cells were added and were allowed to settle for 15 min at 4°C, followed by a 30-min incubation at 37°C. Subsequently, nonadherent T cells were removed by washing with warm Iscove's medium. The adherent cells were lysed with 100 $\mu$l of 1% Triton X-100 and radioactivity was quantified. Results are expressed as the mean per cent of cells binding from triplicate wells. Binding to control L cells was always <10%.

### Radiolabeling and Immunoprecipitation

Before iodination cells were pretreated with 5 mM EDTA for 15 min at RT, cells were washed with cation-free medium and were surface labeled with Na$^{125}$I (Amersham International, Amersham, UK) through the lactoperoxidase method (van Kooyk et al., 1991). For immunoprecipitation, JS136 (20 × 10$^6$) were lysed for 1 h at 4°C in immunoprecipitation buffer (IPB) which contained 1% NP-40, 50 mM Triethanolamine, pH 7.4, 150 mM NaCl and as protease inhibitors, 1 mM PMSF, ovomucoid trypsin inhibitor (0.02 mg/ml), 0.02 mg/ml leupeptin, and 1 mM Nd-P-Tosyl-L-lysine chloromethyl ketone were added. The IPB contained 5 mM EDTA, or 2 mM cations as depicted in Fig. 1 B. Nuclear debris was removed from the lysates by centrifugation at 13,000 g for 15 min at 4°C. Lysates were precleared with mouse IgG covalently coupled to protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ). Precleared lysates were incubated for 2 h with NKI-L16 or SPV-L7 coupled to protein A-Sepharose. The immunoprecipitates were removed from the lysates by centrifugation, and washed with IPB containing 5 mM EDTA or 2 mM cations. Samples were analyzed under reducing conditions with 5% 2-mercaptoethanol in SDS sample buffer on SDS-page on acrylamide gels (5–15%). Dried gels were exposed to Kodak XAR-film (Eastman Kodak Co., Rochester, NY). Molecular weight markers are depicted.

## Results

### The L16 Epitope Is a $Ca^{2+}$ Binding Reporter

It has been previously described that NKI-L16 antibodies recognize a unique epitope located on the $\alpha$ chain of LFA-1, and that binding of this antibody stimulates LFA-1–mediated adhesion. Expression of the L16 epitope is $Ca^{2+}$ dependent, and epitope expression is required for LFA-1 mediated adhesion (van Kooyk et al., 1991). Here we further analyzed the role of other divalent cations on L16 epitope expression using T cell clones (JS136 is used as a prototype T cell clone throughout this study), which express high levels of the L16 epitope. Treatment of JS136 with EGTA or EDTA (to specifically remove $Ca^{2+}$ or all divalent cations, respectively) completely removes the L16 epitope from LFA-1. Expression of other epitopes on the $\alpha$ chain of LFA-1 (SPV-L7), or the $\beta$-chain (not shown) remain unchanged (Table I; Fig. 1 A).

**Table I.** *Effect of Divalent Cations on the Expression of the L16 Epitope After EDTA Treatment*

| Treatment JS136 | Relative fluorescence intensity | | |
|---|---|---|---|
| | Control | SPV-L7 | NKI-L16 |
| Medium | 5 | 373 | 300 |
| EGTA | 5 | 310 | 21 |
| EDTA* | 5 | 290 | 30 |
| +Mg$^{2+}$ | 5 | 417 | 19 |
| +Ca$^{2+}$ | 7 | 335 | 300 |
| +Mn$^{2+}$ | 6 | 251 | 31 |
| +Co$^{2+}$ | 6 | 360 | 20 |
| +Zn$^{2+}$ | 8 | 335 | 22 |
| +Cu$^{2+}$ | 9 | 311 | 34 |
| +Sr$^{2+}$ | 9 | 320 | 306 |
| +Ni$^{2+}$ | 8 | 307 | 31 |

* JS136 T cells were pretreated with 5 mM EDTA for 15 min at 37°C, to remove all cell-bound divalent cations. Subsequently, cells were washed twice with cation-free medium, followed by the addition of distinct divalent cations (1 mM). Cells were incubated with NKI-L16 or SPV-L7 antibodies for 30 min at 4°C, were washed and incubated for 30 min with FITC-labeled antibody. Fluorescence is measured in the FACScan, expression is depicted as relative fluorescence intensity. One experiment out of three is shown.
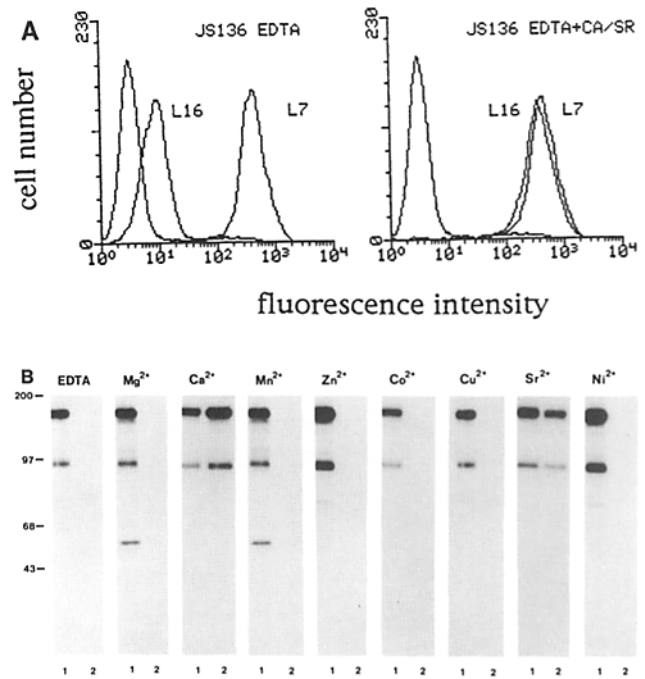


*Figure 1.* Effect of Ca$^{2+}$ or Sr$^{2+}$ on the expression of the L16 epitope on LFA-1 after EDTA treatment of JS136 T cells. Cells were treated with 5 mM EDTA, washed twice with cation free medium, and restored with 1 mM Ca$^{2+}$ or Sr$^{2+}$, which give the same facs profile. (*B*) Effect of cations on immunoprecipitation of LFA-1 from $^{125}$I-labeled lysate of JS136 T cells with two different anti–LFA-1 mAbs SPV-L7 (lanes *1*) and NKI-L16 (lanes *2*). JS136 T cells were pretreated with 5 mM EDTA. Immunoprecipitation was performed in the presence of 5 mM EDTA, or upon restoration of 2 mM divalent cations. Bars indicate relative molecular mass (kD) as defined by prestained markers. A representative experiment out of three is shown.

We observed that in addition to Ca$^{2+}$, also Sr$^{2+}$ can restore expression of the L16 epitope, whereas Mg$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Mn$^{2+}$, or Ni$^{2+}$ can not. Immunoprecipitation studies (Fig. 1 *B*) also revealed that mAb NKI-L16 (lane *2*) could only precipitate LFA-1 when Ca$^{2+}$ or Sr$^{2+}$ are present, whereas precipitation by a regulator anti–LFA-1 mAb (SPV-L7, lane *1*) was independent on the presence of cations. This indicates that mAb NKI-L16 only recognizes the Ca$^{2+}$ or Sr$^{2+}$ LFA-1 complex.

### Mg$^{2+}$ Does Not Inhibit Ca$^{2+}$ Binding to Form the L16 Epitope

Since Mg$^{2+}$ plays an important role in LFA-1–mediated adhesion (Dransfield et al., 1989, 1992), we next investigated if Mg$^{2+}$ ions can displace Ca$^{2+}$, and thus prevent the L16 expression (Table II). After removal of both cell bound Ca$^{2+}$ and Mg$^{2+}$ with EDTA, cation concentrations were restored according to a specific sequence. Addition of Mg$^{2+}$ before Ca$^{2+}$ (or vice versa; Ca$^{2+}$ followed by Mg$^{2+}$) did not inhibit the formation of the L16 epitope, and thus could not prevent Ca$^{2+}$, nor Sr$^{2+}$ (not shown) from binding to LFA-1 to restore the L16 epitope. These results clearly demonstrate that Mg$^{2+}$ binds to a site distinct from the one that binds Ca$^{2+}$ to express the L16 epitope.

### Strong or Weak Binding of Ca$^{2+}$ to LFA-1

We noticed, by determining the cation dependency of the L16 epitope on a number of other T cells, such as the T cell line Peer and resting peripheral blood lymphocytes (PBL), that the expression of this epitope is directly related to the concentration of Ca$^{2+}$ or Sr$^{2+}$ ions in the medium. Surprisingly, only washing of resting T cells or Peer T cells in cation free buffer, lowered the L16 expression dramatically, compared to the expression found in the presence of complete medium (1 mM Mg$^{2+}$ and 1 mM Ca$^{2+}$) (Fig. 2; Table III). In contrast, washing of JS136 T cells in cation free buffer hardly affected the L16 expression (Fig. 2; Table III). Similar results were obtained with a number of other T cell clones (not

shown). These data indicate that LFA-1 expressed on different T cells bind Ca$^{2+}$ ions with different strength: Ca$^{2+}$ (Peer T cells, resting PBL) which is easily removed by washing with Ca$^{2+}$-free medium (low affinity) and Ca$^{2+}$ (JS-36 T cells) which can only be removed with chelating agents such as EGTA (high affinity).

**Table II.** *Mg$^{2+}$ Does Not Inhibit Binding of Ca$^{2+}$ to Form the L16 Epitope*

| Treatment JS136 | | | Relative fluorescence intensity | | |
|---|---|---|---|---|---|
| First | Second | Third | Control | SPV-L7 | NKI-L16 |
| EDTA | - | - | 5 | 329 | 27 |
| EDTA | Ca$^{2+}$ | - | 5 | 345 | 327 |
| EDTA | Ca$^{2+}$ | Mg$^{2+}$ | 7 | 360 | 340 |
| EDTA | Mg$^{2+}$ | - | 5 | 333 | 26 |
| EDTA | Mg$^{2+}$ | Ca$^{2+}$ | 5 | 351 | 314 |

JS136 T cells were pretreated with 5 mM EDTA (first) for 15 min at 37°C, cells were washed twice with cation free medium. Subsequently cells were incubated with distinct divalent cations (second), 1 mM Mg$^{2+}$, or Ca$^{2+}$. After 15 min at 4°C the other divalent cations (1 mM) were added, together with the antibodies (third treatment). Antibodies were incubated for 30 min at 4°C, cells were washed and incubated with FITC-labeled antibody for 30 min at 4°C. Expression is depicted as relative fluorescence intensity. One experiment out of three is shown.
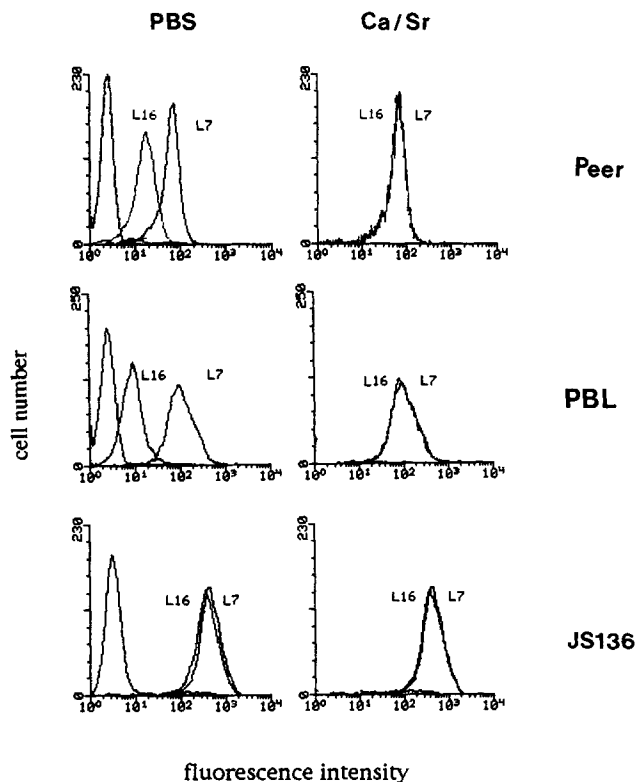
PBS        Ca/Sr

**Figure 2.** Effect of Ca$^{2+}$ and Sr$^{2+}$ (the same facs profile) on the expression of LFA-1 after washing different T cells (Peer, PBL, JS136) in cation-free buffer.

**Table III. Effect of Divalent Cations on the Expression of the L16 Epitope After Washing in Cation-Free Medium**

| Treatment | Relative fluorescence intensity | | | |
|---|---|---|---|---|
| | Control | SPV-L7 | NKI-L16 | F10.2 |
| JS136 | | | | |
| Medium | 7 | 356 | 320 | 121 |
| Cation free | 6 | 360 | 250 | 143 |
| +Mg$^{2+}$ | 7 | 410 | 237 | 130 |
| +Ca$^{2+}$ | 5 | 314 | 340 | 144 |
| +Mn$^{2+}$ | 6 | 361 | 260 | 136 |
| +Zn$^{2+}$ | 7 | 363 | 207 | 118 |
| +Co$^{2+}$ | 7 | 333 | 221 | 126 |
| +Cu$^{2+}$ | 6 | 259 | 239 | 110 |
| +Sr$^{2+}$ | 5 | 416 | 339 | 133 |
| +Ni$^{2+}$ | 6 | 373 | 225 | 121 |
| Peer | | | | |
| Medium | 2 | 137 | 121 | 25 |
| Cation free | 3 | 132 | 25 | 30 |
| +Mg$^{2+}$ | 3 | 131 | 25 | 27 |
| +Ca$^{2+}$ | 2 | 133 | 121 | 40 |
| +Mn$^{2+}$ | 2 | 135 | 28 | 38 |
| +Zn$^{2+}$ | 2 | 131 | 25 | 34 |
| +Co$^{2+}$ | 2 | 128 | 25 | 25 |
| +Cu$^{2+}$ | 3 | 131 | 25 | 32 |
| +Sr$^{2+}$ | 2 | 132 | 127 | 33 |
| +Ni$^{2+}$ | 2 | 136 | 23 | 25 |

T cells were washed twice with cation free medium. Cells were incubated with antibodies together with divalent cations (1 mM) for 30 min at 4°C and were washed once followed by incubation with FITC-labeled antibody for 30 min at 4°C. One experiment out of four is shown.
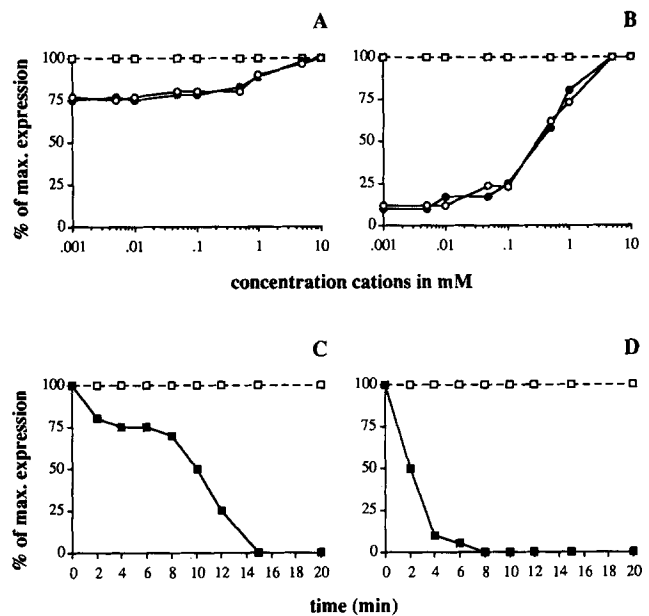


**Figure 3.** Removal of the L16 epitope expressed on JS136 T cells (A and C) and Peer T cells (B and D) upon washing in cation free buffer (A and B) or upon EDTA treatment (C and D). T cells (JS136, A; Peer, B) were washed twice in cation free buffer to remove low affinity Ca$^{2+}$ bound to LFA-1. Subsequently cells were washed twice and were incubated with different concentrations of Sr$^{2+}$ (o) or Ca$^{2+}$ (●) (ranging from 0.001 to 10 mM) together with the first antibody (NKI-L16 or SPV-L7) for 30 min at 4°C. T cells (JS136, C; Peer, D) were incubated for different time periods with 5 mM EDTA, after which the L16 expression was determined at different time points in the absence of cations (■). Cells were washed and incubated with the FITC-labeled antibody for 30 min at 4°C. Values are expressed as percent of maximal expression of the L16 epitope in medium, which is equal to the L7 (□) epitope expression. One experiment out of two is shown.

We defined the optimal concentration of Ca$^{2+}$ and Sr$^{2+}$ required to restore the L16 epitope after removal of the cations by washing T cells in cation free buffer (Fig. 3 A, JS136 cells and 3B Peer T cells). 0.5–1 mM Ca$^{2+}$ or Sr$^{2+}$ could restore the L16 expression to a level similar as observed with SPV-L7 antibodies. The results show that in the presence of low (0.1 mM) extracellular Ca$^{2+}$ concentrations, Peer T cells do not express the L16 epitope (low affinity), whereas the epitope remains expressed on JS136 T cells under these conditions (high affinity). Treatment of JS136 and Peer T cells for different time periods with chelating agents like EDTA (Fig. 3, C and D, respectively) shows that the L16 epitope is rapidly lost from Peer (within 1 min), whereas 10–15 min are required to remove the L16 epitope of JS136. These results again demonstrate that Ca$^{2+}$ is differentially bound by LFA-1 on these cells, and suggest that Ca$^{2+}$ can be bound with different affinities.

Interestingly, we observed that NKI-L16 antibodies can protect Ca$^{2+}$ from removal of LFA-1. After binding of the NKI-L16 antibody in the presence of Ca$^{2+}$ or Sr$^{2+}$, washing of the Peer T cells with cation free buffer did not affect expression of the L16 epitope, whereas EDTA or EGTA treatment removed the L16 epitope completely (Table IV).

We conclude from these results that Ca$^{2+}$ can be bound by LFA-1 with high and low affinity, and that binding of NKI-

**Table IV. NKI-L16 Antibodies Protect $Ca^{2+}$ from Being Removed from LFA-1 by Incubation in Cation-Free Medium**

| Treatment Peer | | |
|---|---|---|
| First | Second | Fluorescence intensity |
| Control | Medium | 3 |
| Control | Cation free | 3 |
| Control | EDTA | 3 |
| Control | EGTA | 3 |
| SPV-L7 | Medium | 142 |
| SPV-L7 | Cation free | 137 |
| SPV-L7 | EDTA | 132 |
| SPV-L7 | EGTA | 140 |
| NKI-L16 | Medium | 130 |
| NKI-L16 | Cation free | 122 |
| NKI-L16 | EDTA | 5 |
| NKI-L16 | EGTA | 7 |

Peer T cells were preincubated in cation containing medium with the first antibody (control, SPV-L7, or NKI-L16) for 30 min at 4°C. Subsequently cells were washed with cation containing medium, cation free buffer or EDTA (5 mM) and incubated for 15 min at RT with the same buffer. Finally cells were washed with PBA, and FITC-labeled antibody was added for 30 min at 4°C. Expression is indicated as relative fluorescence intensity. One experiment out of four is shown.

L16 antibody protects low affinity bound $Ca^{2+}$ from removal of LFA-1.

## Role of $Ca^{2+}$ in LFA-1-Mediated Adhesion

We next investigated whether binding of $Ca^{2+}$ had any effect on LFA-1 mediated adhesion (Fig. 4). If Peer or JS136 cells are suspended in medium that contains $Mg^{2+}$ and $Ca^{2+}$, both cell types bind to L-ICAM-1 (L cells transfected with ICAM-1 cDNA) upon activation of LFA-1 by PMA or NKI-L16 (Fig. 4). Binding of the cells is ICAM-1 specific since no binding is observed to control (mock-transfected) L cells. After washing of the cells with cation free buffer neither PMA nor NKI-L16 can induce ICAM-1 binding of JS136 as well as Peer T cells. Addition of $Mg^{2+}$ (1 mM) restores only binding of JS136 to ICAM-1 when LFA-1 is activated. Whereas no binding to Peer T cells was observed after stimulation with PMA or NKI-L16 (Fig. 4). These data correspond with the finding that LFA-1 of JS136 cells still contains $Ca^{2+}$ (bound with high affinity), whereas Peer cells lost the low affinity bound $Ca^{2+}$ (Fig. 3). Only if both $Mg^{2+}$ and $Ca^{2+}$ levels are restored, binding of Peer cells to ICAM-1 is observed. These data provide further evidence that not only $Mg^{2+}$ but also $Ca^{2+}$ is important for LFA-1 mediated adhesion, however restoration of $Ca^{2+}$ alone is not sufficient to restore binding of JS136 or Peer. Furthermore we conclude that $Mg^{2+}$ does not bind to LFA-1 with high affinity unless activated.

We consistently observed that after induction of adhesion by PMA or NKI-L16 antibodies in the presence of extracellular $Ca^{2+}$ and $Mg^{2+}$, the capacity of JS136 T cells to bind to ICAM-1 was always higher than that of Peer T cells (Fig. 4) or resting PBL (not shown). Surprisingly, Peer T cells showed a significant increase in adhesion to ICAM-1 when both stimuli (PMA and L16) were combined. Since the results in Table IV demonstrate that binding of NKI-L16 antibodies stabilize binding of $Ca^{2+}$ to LFA-1 on Peer T cells, LFA-1 is likely converted into a form similar to that found
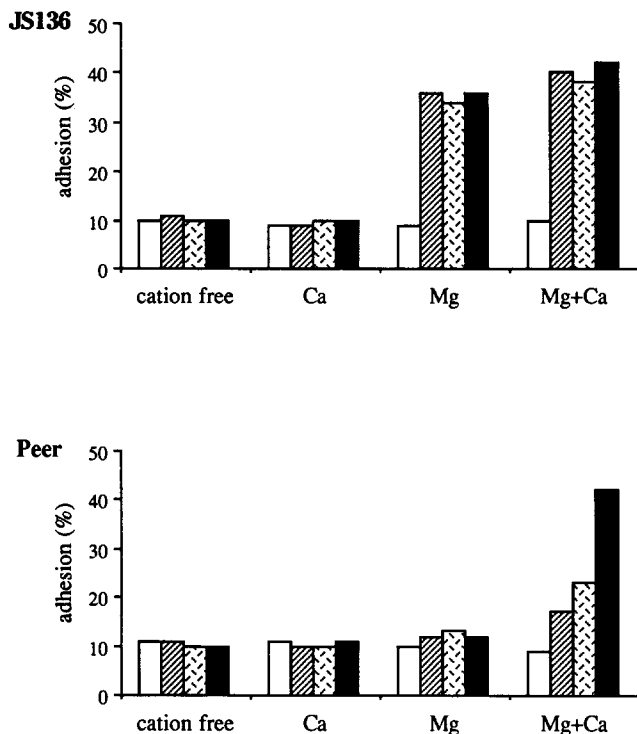


*Figure 4.* PMA and NKI-L16 induced adhesion of JS136 cells and Peer T cells to ICAM-1 transfected L cells. L-ICAM-1 cells were cultured to obtain a monolayers of cells. $^{51}$Cr-labeled T cells were preincubated without stimulus (□), with PMA (▨) (50 ng/ml), NKI-L16 antibodies (▣) (10 mg/ml), or a combination of both (■) (10 μg/ml) for 15 min at 4°C. The adhesion assay was performed in medium containing no cations, 1 mM $Mg^{2+}$, 1 mM $Ca^{2+}$, or a combination of both for 30 min at 37°C. The mean of two experiments is shown (SD < 3%).

on JS136 in which $Ca^{2+}$ is bound with high affinity. This enables Peer T cells to respond to PMA and increases LFA-1 mediated adhesion (Fig. 4).

## L16 Expression Is Associated with a Clustered Cell Surface Distribution of LFA-1

Since previous observations by Detmers et al. (1987) showed a correlation between clustering of β2 integrins at the cell surface and cell adhesion, we investigated whether differences in the affinity of LFA-1 for extracellular $Ca^{2+}$ affect the cell surface distribution of LFA-1 (Figs. 5–7). Using confocal laser microscopy we observed that Peer T cells showed a dispersed LFA-1 distribution, consisting of numerous small aggregates. In contrast, LFA-1 on JS136 T cells was localized into large clusters, when stained with anti-LFA-1α Fab fragments (SPV-L7) (Fig. 5). Double labeling of cells with SPV-L7 and NKI-L16 antibodies showed the same distribution, thus the L16 epitope was always found in the clustered LFA-1 (not shown). Washing of JS136 T cells in cation free buffer, hardly changed the distribution of LFA-1. To determine if the L16 epitope, and thereby binding to $Ca^{2+}$ ions, are necessary for clustering of LFA-1, JS136 T cells were pretreated with EGTA or EDTA to remove $Ca^{2+}$ from LFA-1. This dramatically changed the clustered distribution of LFA-1 from large clusters into a more dispersed distribution, indicating
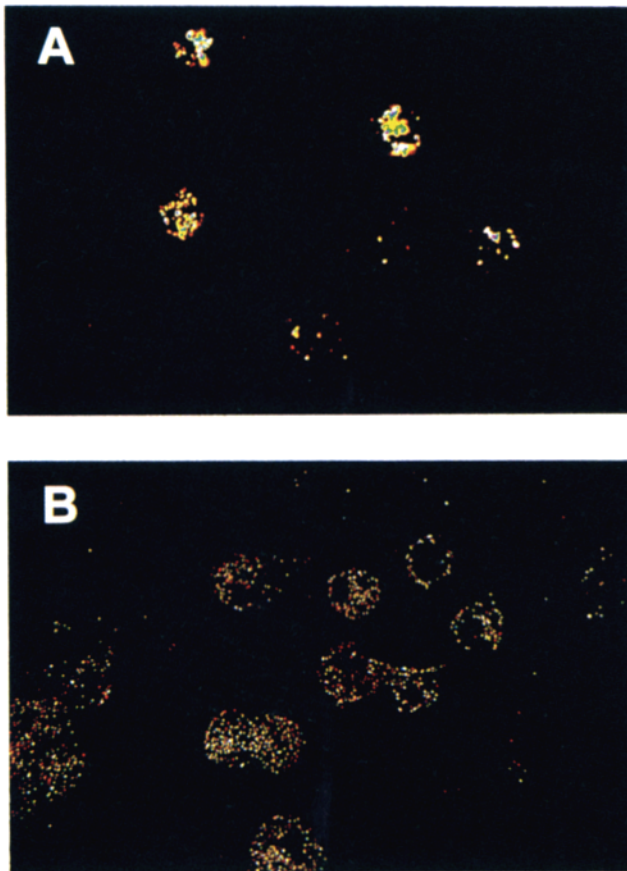
*Figure 5.* Overview of the distribution of LFA-1 on different T cells. Distribution of LFA-1 was determined by CLSM, using SPV-L7 Fab fragments. LFA-1 is localized in large clusters on JS136 in the presence of medium (*A*), while it is dispersed present on Peer T cells (*B*).

that binding of $Ca^{2+}$ to LFA-1 is critically important to cluster LFA-1 receptors at the cell surface (Fig. 6). Moreover, reconstitution with $Ca^{2+}$ but also with $Sr^{2+}$ ions, restored the clustered LFA-1 distribution (Fig. 7). Notably, neither $Mg^{2+}$ (Fig. 7) nor any of the other cations (not shown) were able to restore receptor clustering, again pointing to different roles of $Ca^{2+}$ and $Mg^{2+}$ in LFA-1 mediated adhesion.

Quantitative data on the relative size of the spots of LFA-1 at the cell surface were obtained using a digital image analyser. Note that it is not possible to measure individual LFA-1 molecules by this technique since the resolution of the visible light is limited to about 0.5 $\mu$m, which equals ~250 molecules, considering the predicted size of an individual integrin, i.e., 20 nm (Calvete et al., 1992). Still, a relative size distribution of the spots can be given. We observed that the size of most LFA-1 spots on JS136 T cells is 30-fold larger (from 100 to 3300) in the presence of medium compared to the spots measured after EGTA (Fig. 8) or EDTA (not shown) treatment. Reconstitution with 2 mM $Ca^{2+}$ restores clustering (from 100 to 1,000) within 15 min. Control experiments showed that both chelators did not affect the membrane distribution of several other cell membrane molecules on JS136 T cells, such as MHC class I antigen or ICAM-1, as observed on the images as well as by the quantitative anal-
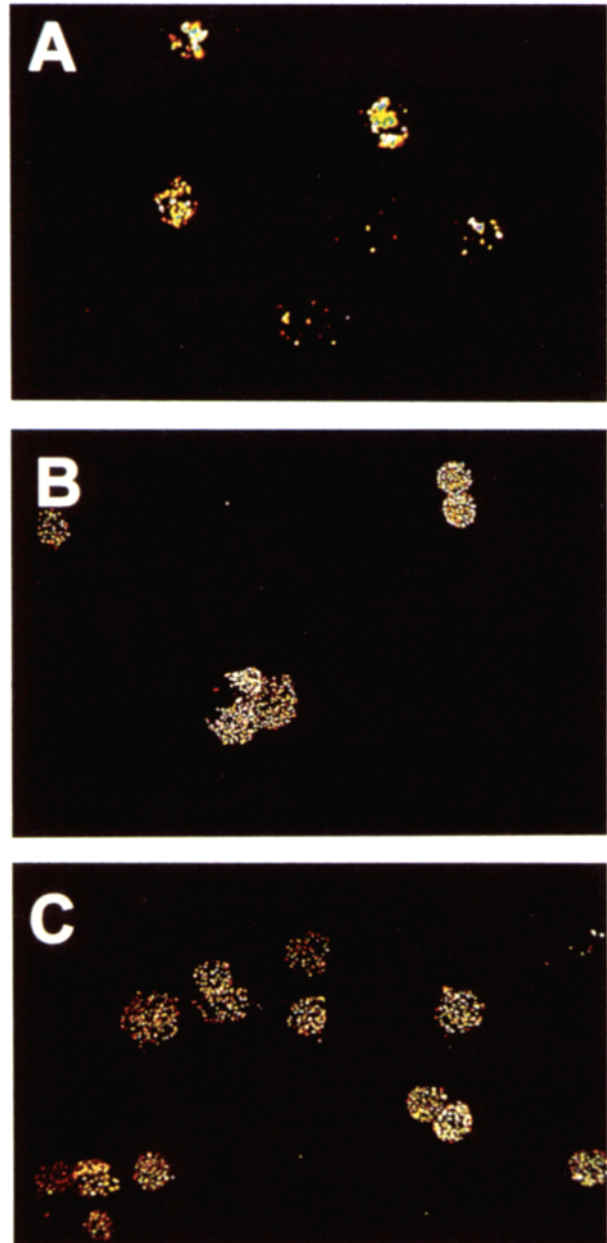
*Figure 6.* Overview of the distribution of LFA-1 on JS136 T cells in the presence of medium (*A*) or after treatment with 5 mM EDTA (*B*) or EGTA (*C*) for 15 min at 37°C resulted in a dispersed LFA-1 distribution. Top views of LFA-1 distribution on JS136 are depicted.

ysis (not shown). These data demonstrate that $Ca^{2+}$ ions, and thus the expression of the L16 epitope directly correlates with the distribution of LFA-1 into clusters. Moreover clustering of LFA-1 on the cell surface facilitates strong interaction of LFA-1 with its ligand (Fig. 4).

## Discussion

From the results the following conclusions can be drawn: (*a*) LFA-1 binds $Ca^{2+}$ ions with different strengths depending on the activation/maturation state of the T cells; (*b*) $Ca^{2+}$ can be replaced by $Sr^{2+}$ ions; (*c*) binding of $Ca^{2+}$ or $Sr^{2+}$ by
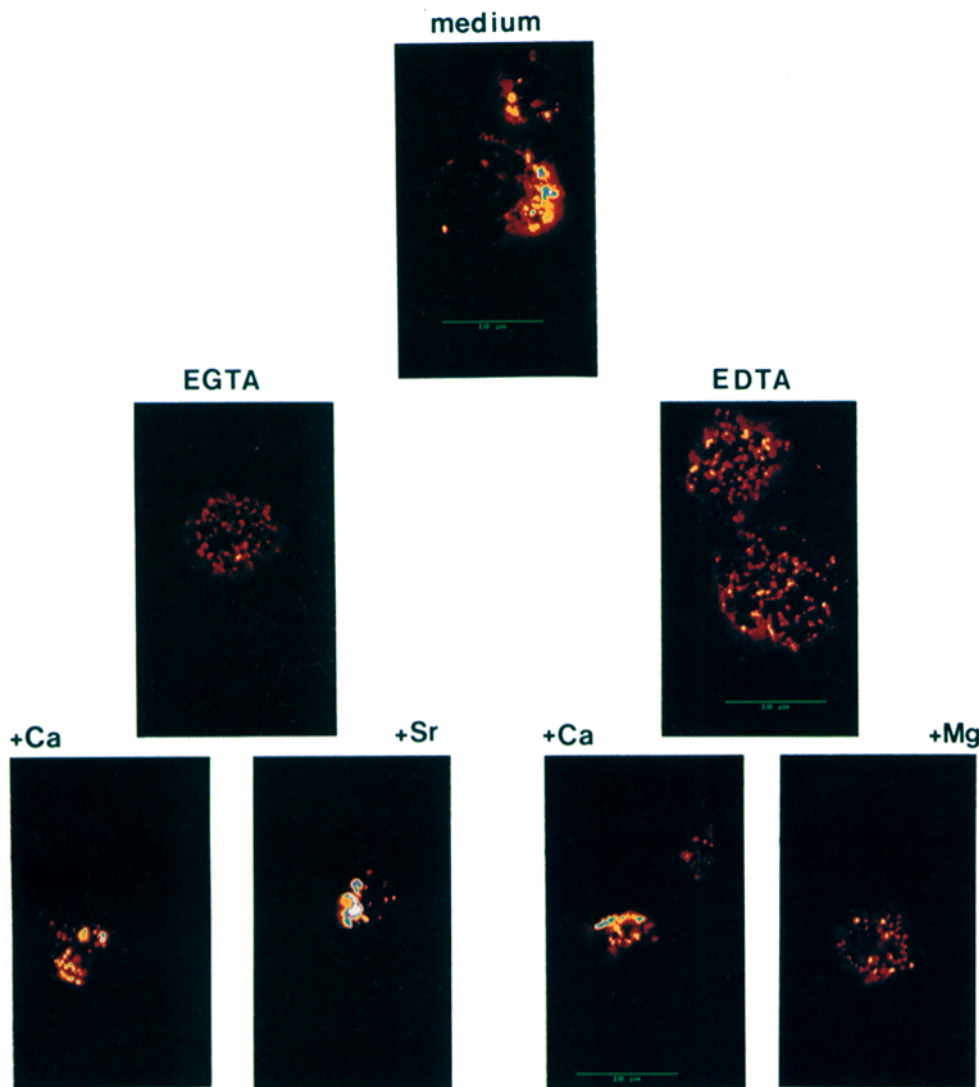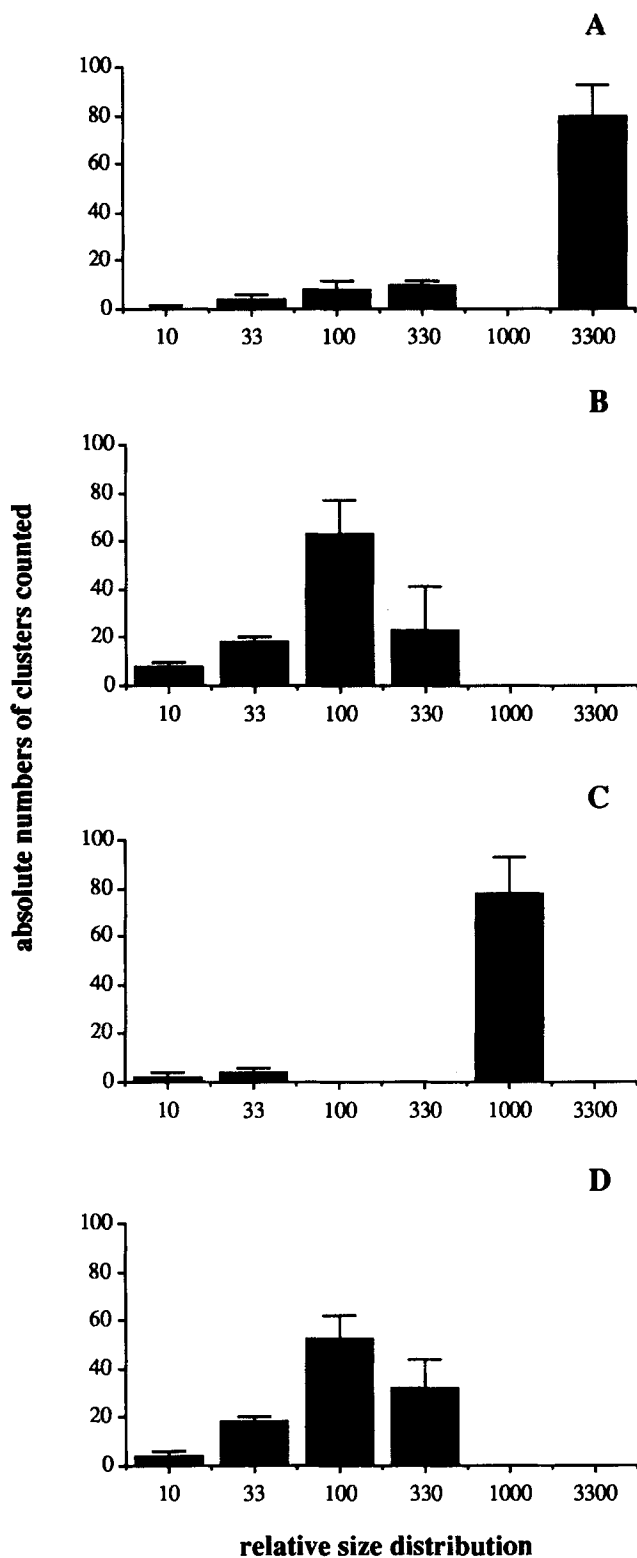
*Figure 7.* Reconstruction of clustered LFA-1 on JS136 T cells after the addition of $Ca^{2+}$ or $Sr^{2+}$ ions. JS136 cells were treated with 5 mM EDTA or EGTA, as described in Fig. 5, to remove all cell bound divalent cations. Subsequently $Ca^{2+}$, $Sr^{2+}$, or $Mg^{2+}$ ions were added and LFA-1 distribution was determined by CLSM, using SPV-L7 Fab fragments. One representative experiment out of four is shown.

LFA-1 correlates with ligand independent clustering of LFA-1 at the cell surface; (*d*) $Ca^{2+}$-mediated clustering of LFA-1 facilitates $Mg^{2+}$-dependent ligand binding; (*e*) $Ca^{2+}$ and $Mg^{2+}$ ions play a different role in LFA-1 mediated adhesion by T cells. $Ca^{2+}$ is involved in avidity regulation of LFA-1 by clustering of LFA-1 molecules at the cell surface, whereas $Mg^{2+}$ is important in regulation of the affinity of LFA-1 for its ligands.

Here we show for the first time that binding of $Ca^{2+}$ ions correlates with ligand-independent clustering of LFA-1 at the cell surface. These results are in line and extend previous observations of Detmers et al. (1987), who showed that the $\beta2$ integrin CR3 is clustered at the cell surface and that clustering correlates with cell adhesion. Binding of $Ca^{2+}$ by LFA-1 (Fig. 1 *B*), and thus L16 epitope expresion, is a reflection of LFA-1 membrane distribution, also since expression of the L16 epitope is always found in clustered LFA-1. We previously reported that LFA-1 cannot be stimulated to bind ligand unless the L16 epitope is expressed (Figdor et al., 1990; van Kooyk et al., 1991). We now presume that strong binding of $Ca^{2+}$ by LFA-1 (high affinity) converts LFA-1 from an inactive- into a "potentially active" form, thus lowering the threshold of LFA-1 to respond to stimuli leading to activation

of LFA-1 (Figdor et al., 1990). In contrast, cells on which $Ca^{2+}$ is only weakly bound to LFA-1 (low affinity) (resting PBL or Peer T cells) can not respond to activating stimuli, indicating that LFA-1 is in an "inactive" state. Nevertheless, we show (Fig. 4) that the capacity of such cells to respond can be restored after binding of NKI-L16 antibodies which apparently stabilize binding of $Ca^{2+}$ to LFA-1. Binding of NKI-L16 may enhance the affinity of LFA-1 for $Ca^{2+}$ or mimic high affinity $Ca^{2+}$ binding (Table IV). This may explain why addition of PMA together with the NKI-L16 antibodies results in further enhancement of LFA-1 mediated adhesion (Fig. 4).

That LFA-1 can bind $Ca^{2+}$ with different affinities is based on the described washing experiments and experiments using chelators. The affinity of $Ca^{2+}$ binding to LFA-1 on resting PBL or Peer T cells must be extremely low, since simple washing with cation free buffer is capable of removing $Ca^{2+}$. Binding of $Ca^{2+}$ to LFA-1 by JS136 cells is of significantly higher affinity, since it is only removed by the chelators EDTA or EGTA. In addition, we observed that $Ca^{2+}$ is removed from LFA-1 on JS136 cells only after 10–15 min, indicating that $Ca^{2+}$ is firmly bound to LFA-1 on these cells. It is not possible to exactly determine the affinities with

A

100
80
60
40
20
0
10   33   100   330   1000   3300

B

100
80
60
40
20
0
10   33   100   330   1000   3300

C

100
80
60
40
20
0
10   33   100   330   1000   3300

D

100
80
60
40
20
0
10   33   100   330   1000   3300

**absolute numbers of clusters counted**

**relative size distribution**

*Figure 8.* Relative quantification of the dotsize distribution of LFA-1 using a digital image analyzer. The relative size of the clusters of LFA-1 was quantified on JS136 T cells in the presence of medium (*A*), after EGTA treatment for 15 min at 37°C (*B*), after reconstitution of the EGTA treated cells with 2 mM $Ca^{2+}$ (*C*) or with 2 mM $Mg^{2+}$ (*D*). The mean of calculation of eight cells is depicted. One representative experiment out of three is shown.

which $Ca^{2+}$ binds to LFA-1 using intact cells since a variety of $Ca^{2+}$ binding proteins are expressed. Furthermore, since the different activation states of LFA-1 do not exist when the receptor is solubilized, it is not possible to perform affinity studies with isolated receptors. Precise characterization of cation binding to LFA-1 awaits crystallography studies.

It is not known what drives the different activation states of LFA-1 expressed on leukocytes. It might be that local cytokine release may invoke the activation/maturation state of the lymphocyte resulting in enhanced $Ca^{2+}$ binding by LFA-1 thus increasing the L16 expression. We previously demonstrated that IL-2 stimulates L16 expression on resting PBL (van Kooyk et al., 1991). The expression of different activation states of adhesion receptors on the cell surface is not restricted to the $\beta2$ integrins, but has also been reported for the $\beta1$ integrins (Masumoto and Hemler, 1993; Faull et al., 1993).

In line with the data of Detmers et al. (1987) we observed that clustering of LFA-1 is independent from ligand binding. This notion is supported by double labeling studies with anti–LFA-1 antibodies and anti–ICAM-1 antibodies. We could exclude co-localisation of ICAM-1 and LFA-1, thus excluding receptor-ligand interactions on one and the same cell (van Kooyk, Y., and G. Figdor, unpublished observations). These results were confirmed by capping experiments, showing independent expression of LFA-1 and ICAM-1. In general, artificial clustering of LFA-1 by anti-LFA-1 mAbs does not mimic clustering of LFA-1 mediated by $Ca^{2+}$, indicating that only $Ca^{2+}$-mediated clustering of LFA-1 receptors most likely increases the avidity of LFA-1–ligand interactions. However, it should be noted that stable ligand binding still requires a $Mg^{2+}$-dependent affinity change within the molecule (Staatz et al., 1989; Martz, 1980; Graham et al., 1991; Elices et al., 1991; Rothlein et al., 1986) induced by signals like PMA, anti-CD2 or anti-CD3 (Dustin et al., 1989; van Kooyk et al., 1989). Therefore, we can conclude that both avidity changes ($Ca^{2+}$) as well as affinity changes ($Mg^{2+}$) within LFA-1 contribute to a stable LFA-1-ligand interaction.

Because of the limiting resolution of visible light (0.5 $\mu$m) we were unable to identify single LFA-1 molecules at the cell surface. Quantification of LFA-1 distribution on JS136 showed large clusters of LFA-1, whereas the aggregates on EDTA or EGTA treated cells were 10–30-fold smaller. The number of LFA-1 molecules within each cluster can still only be guessed. Considering a predicted size of 20 nm for an individual integrin (Calvete et al., 1992), the estimated number of LFA-1 molecules varies between 2,000–8,000 molecules in the largest aggregates and ≤250 molecules in the smallest measurable aggregates.

We observed that apart from $Ca^{2+}$ also $Sr^{2+}$ can form the L16 epitope. Recent crystallographic studies of cation-binding motifs (EF-hands) in parvalbumin demonstrated that divalent cations with an ionic radius closest to 1 Å favors binding to cation-binding motifs (EF-hands) (Declerc et al., 1991). Both $Ca^{2+}$ and $Sr^{2+}$ come closest of all divalent cations to this value, with an ionic radius of 0.94 and 1.10 Å, respectively. Three potential cation binding sites are present on the $\alpha$ chain of LFA-1 (Larson et al., 1989). The finding that both $Ca^{2+}$ and $Sr^{2+}$ can form the L16 epitope suggest that both cations may bind the same domain with high affinity. If is not known how binding of $Ca^{2+}$ or $Sr^{2+}$ to LFA-1 results in clustering of LFA-1. We speculate that $Ca^{2+}$ may

link the α-chain of one α/β heterodimer with the β chain of an adjacent α/β heterodimer. One argument in favor of inter-molecular interactions is the observation of Calvete et al. (1992) that the cation binding domains of integrins may be exposed at the outside of the integrin molecule. Further-more, the importance of $Ca^{2+}$ in α/β complex formation of receptors has also been reported for other integrins (Fitzger-ald et al., 1985; Fujimura et al., 1983).

The finding that the L16 expression is neither enhanced nor decreased upon activation of the LFA-1 receptor by CD2, CD3 triggering or PMA, indicates that this $Ca^{2+}$ binding site is also occupied upon ligand binding. This is opposite to the expression of the 24 epitope, a $Mg^{2+}$ dependent epi-tope on LFA-1, which parallels functional activity of LFA-1 (Dransfield et al., 1992). A negative regulatory role for $Ca^{2+}$ in integrin activation has been reported (Dransfield et al., 1992; Staatz et al., 1989; Kirchhofer et al., 1991; Dransfield, 1991; Ginsberg et al., 1991). The data described here, in which $Mg^{2+}$ ions do not inhibit $Ca^{2+}$ binding and expression of the L16 epitope, are distinct from the observa-tion that $Ca^{2+}$ has an inhibitory effect on the expression of the $Mg^{2+}$-dependent epitope as recognized by the antibody 24 (Dreyer et al., 1991; Dransfield et al., 1992). Together, these findings strongly suggest that L16 and 24 recognize dis-tinct cation binding domains. This notion is supported by the finding that $Ca^{2+}$ occupancy, as detected by NKI-L16 bind-ing, can be replaced by $Sr^{2+}$, whereas $Mg^{2+}$ occupancy, as detected by 24 binding, can be inhibited by $Ca^{2+}$ but not by $Sr^{2+}$ (Dransfield et al., 1992). Further evidence for the no-tion that both antibodies recognize distinct sites on LFA-1 comes from the finding that $Mg^{2+}$ does not affect binding of $Ca^{2+}$ to form of the L16 epitope (Table II), and that L16 is exclusively expressed by LFA-1, whereas the epitope recog-nized by antibody 24 is expressed by all three β2 integrins. Because anti-CD18 antibodies block binding of 24 antibod-ies, it has been suggested that residues on the β subunit might participate with the proposed cation binding sequences on the α subunit to provide the sixth co-ordination site (-Z) for $Mg^{2+}$ binding (Dransfield, 1991). However, since the L16 epitope is not blocked by anti-CD18 antibodies, and since ex-pression of L16 does not correspond with ligand bound LFA-1, it is likely that the β-chain is not involved in the formation of the $Ca^{2+}$ binding site that may form the L16 epitope. In relation to this, the recent finding of Michishita et al. (1993), who describe a novel cation-binding site located in the A-domain of the α chain of CR3 nicely demonstrate that divalent cations like $Mn^{2+}$ and $Mg^{2+}$ can bind to this new divalent cation-binding site, which is also shown to be im-portant in ligand binding.

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