Low-affinity LFA-1/ICAM-3 interactions augment LFA-1/ICAM-1-mediated T cell adhesion and signaling by redistribution of LFA-1

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SUMMARY

Although ICAM-3 is implicated in both adhesion and signal transduction events of leukocytes, its low affinity for LFA-1 compared to other ligands of LFA-1 has puzzled many investigators. Here we investigated the role of ICAM-3 in supporting LFA-1-mediated ICAM-1 binding and subsequently cell signaling. We observed that although ICAM-3 binds poorly to LFA-1 expressed on resting T cells, it specifically facilitates and increases LFA-1-mediated adhesion to the high affinity ligand of LFA-1, ICAM-1. We demonstrate that low-affinity binding of LFA-1 to ICAM-3 together with ICAM-1 alters the cell surface distribution of LFA-1 dramatically, inducing large clusters of LFA-1 that facilitate ICAM-1 binding after LFA-1 activation. We found that LFA-1-mediated ICAM-1 cell-cell interactions such as T cell proliferation greatly depend on low affinity LFA-1/ICAM-3 interactions that enhance stable LFA-1/ICAM-1 cell-cell contact. Taken together, these data demonstrate that low affinity LFA-1 binding to ICAM-3 regulates strong LFA-1/ICAM-1-mediated adhesion by driving LFA-1 into clusters to facilitate cell-cell interactions that take place in the immune system.

Key words: Adhesion molecule, T lymphocyte, Cell-cell interaction, Co-stimulatory molecule, Signal transduction

INTRODUCTION

The integrin LFA-1 (CD11a/CD18) is a leukocyte adhesion receptor that coordinates different adhesive events in the immune system. LFA-1 plays an important role in the initiation of immune responses by establishing contact between antigen presenting cells and T cells, as well as in effector functions such as CTL/target interactions, T cell proliferation, and migration of leukocytes through endothelial cell layers into inflamed tissue (Krensky et al., 1983; Martz, 1987; Springer, 1990; Figdor et al., 1990; van Kooyk and Figdor, 1993; van Kooyk et al., 1993, 1994; Mackay, 1995; Imhof and Dunon, 1995; Lub et al., 1995, 1997a,b; Stewart and Hogg, 1996; Kucik et al., 1996). LFA-1 (CD11a/CD18) belongs to the β2 family of integrins, which share a common β subunit (CD18), but have distinct but structurally homologous α subunits. Other members of the β2-family are Mac-1 (CD11b, αM/β2), p150,95 (CD11c, αc/β2) and α5 (CD11d, α5/β2) (Springer, 1990; Sanchez-Madrid et al., 1983; Vandervieren et al., 1995). LFA-1 mediates cell-cell adhesion upon binding to one of the Ig-superfamily members ICAM-1 (Marlin and Springer, 1987; Staunton et al., 1988; Simmons et al., 1988), ICAM-2 (Staunton et al., 1989), or ICAM-3 (Vazeux et al., 1992; Fawcett et al., 1992; de Fougerolles et al., 1993), which have 5, 2, and 5 Ig-like domains, respectively. ICAM-1 is expressed on many cell types, including lymphocytes and activated endothelial cells in inflamed tissue (Dustin et al., 1986). ICAM-2 is present on lymphocytes, endothelial cells and platelets (de Fougerolles et al., 1991; Diacovo et al., 1994), whereas ICAM-3 is only expressed on leukocytes, with the exception of endothelial cells in certain tumors (Acevedo et al., 1993). In contrast to ICAM-1, expression of ICAM-2 and ICAM-3 is not enhanced by cytokines (Acevedo et al., 1993; Cordell et al., 1994).

LFA-1-mediated cell adhesion requires activation of LFA-1 (Hynes, 1992; van Kooyk et al., 1989; Dustin and Springer, 1989; Lub et al., 1995) and is dependent on the presence of divalent cations, such as Mg2+ and Ca2+ (Dransfield et al., 1992), an intact cytoskeleton, and a physiological temperature (Figdor et al., 1990). Activation can be induced by intracellular signals generated upon crosslinking of surface receptors like the TCR/CD3 complex (van Kooyk et al., 1989; Dustin and Springer, 1989), or upon addition of phorbol esters that directly activate protein kinase C (Rothlein and Springer, 1986). LFA-1 can also be activated directly by adding activating antibodies directed against the extracellular domains of the α or β2 subunit (Andrew et al., 1993; Robinson et al., 1992; van Kooyk et al., 1991; Landis et al., 1993). Activation of LFA-1 and subsequent ligand binding is thought to result from conformational changes in the αβ heterodimer that increase both the avidity and affinity of LFA-1 for its ligands (Binnerts and van Kooyk, 1999).

Several reports have illustrated that the cell surface distribution of LFA-1 is also important for its adhesive
function. Expression of a Ca²⁺-dependent epitope on LFA-1 has clearly been shown to correlate with the clustering status of LFA-1 on the cell surface and to facilitate ICAM-1 binding (van Kooyk et al., 1994; Binnerts and van Kooyk, 1999). In addition, disconnecting LFA-1 from the actin cytoskeleton, through inhibitors such as cytochalasin D, or upon deletion of the α and β cytoplasmic tails, have been shown to result in clustering of LFA-1 on the cell membrane and to enhance its avidity and adhesive capacity (Lub et al., 1997a,b; Stewart and Hogg, 1996; Kucik et al., 1996; van Kooyk et al., 1999).

It has been clearly demonstrated that engagement of LFA-1 on T cells by purified ICAM-1, -2, or -3 molecules can co-stimulate T cell proliferation (Bleis et al., 1999; de Fougerolles and Springer, 1992; van Kooyk et al., 1996; Starling et al., 1995). Several reports suggest that ICAM-3 may also function as signal transduction molecule. Crosslinking of ICAM-3 induces protein tyrosine phosphorylation, elevation of intracellular calcium levels, co-stimulation of T cells, morphological changes in T cells, and can lead to activation of B1 and B2 integrins (Juan et al., 1994; Arroyo et al., 1994; Hernandez-Caselles et al., 1993; Campanero et al., 1993; Cid et al., 1994; Delpozo et al., 1994). Whether physiological engagement of ICAM-3 by LFA-1 is important for adhesion/cell signaling is still completely unknown.

Because ICAM-3 is the dominantly expressed ICAM molecule on resting lymphocytes, it was originally proposed to be the primary LFA-1 ligand during the initiation of an immune response (de Fougerolles and Springer, 1992). However, later reports showed that even LFA-1 expressed on activated T cells binds poorly to ICAM-3 when compared to ICAM-1 and ICAM-2 (de Fougerolles et al., 1994; Binnerts et al., 1994, 1996). Despite the low affinity binding of ICAM-3 to LFA-1, blocking antibodies directed against ICAM-3 have been shown to inhibit T cell proliferation in mixed lymphocyte reactions, suggesting that LFA-1/ICAM-3 interaction is important in adhesion and cell signaling of resting T cells (Starling et al., 1995). In this study we investigated whether ICAM-3 contributes directly to LFA-1-mediated adhesion of resting T cells, or is involved in the regulation of LFA-1 function on these cells. We obtained evidence that, although ICAM-3 binds poorly to LFA-1, low-affinity interactions of LFA-1 with ICAM-3 are required for LFA-1/ICAM-1 dependent adhesion and signal transduction during the initiation of an immune response.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: SPV-L7, NKL-L15 (Keizer et al., 1985), YTH81.5 (Landis et al., 1994) and TS2/4 (Sanchez-Madrid et al., 1982), directed against CD11a, T3b directed against CD3 (Spits et al., 1983), REK-1 (Binnerts et al., 1994) and RR1/l (Rothlein et al., 1986) directed against ICAM-1, CBR-IC2/2 (de Fougerolles et al., 1991) directed against ICAM-2, 186-269 and AZN-IC3.1 (6th International Leukocyte Workshop 1996) directed against ICAM-3, and TS2/9 (Sanchez-Madrid et al., 1982) directed against LFA-3. For activation of LFA-1, the LFA-1 activating mAbs KIM185 (Andrew et al., 1993) and MEM-48 (Binnerts et al., 1994), directed against CD18 were used. LFA-1 activation was detected using the activation-dependent-epitope antibodies NKL-L16 against CD11a (Keizer et al., 1988) and M24 against CD11a,b,c kindly provided by Dr N. Hogg (Dransfield and Hogg, 1989). For T cell subsets the following antibodies were used: RIV-6 against CD4 (Leerling et al., 1990), wt82 against CD8 (Tax et al., 1984), anti-CD45RA and anti-CD45RO (Becton and Dickinson & Co., Oxnard, CA).

Cells

The T cell lines Peer and HS6-2 were cultured in Iscove’s medium (Gibco, Life Technologies Ltd, Paisley, Scotland) supplemented with 5% FCS and 1% antibiotics/antimycotics (Gibco). Resting T cells were obtained by centrifugal elutriation of PBMC from blood or bone marrow of healthy donors, as described (Fidgior et al., 1984). The T cell fractions were obtained using more than 90% CD3 positive cells and were cultured in RPMI 1640 (Gibco), supplemented with 10% FCS and 1% antibiotics/antimycotics (Gibco). K562-LFA-1 transfectants were generated as described previously (Lub et al., 1997b) and were grown in RPMI/Iscove’s (75:25) (Gibco) containing 7.5% FCS and genetin (Gibco; 2 mg/ml).

Adhesion assay

96-well (flat bottom) plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with goat anti-human Fc antibodies (4 mg/ml; 50 ml/well; Jackson Immunoresearch Laboratories, Inc., Westgrove, PA) in TSM (150 mM NaCl, 10 mM Tris/HCl, 1 mM MgCl₂, 1 mM CaCl₂, pH 8.0), for 60 minutes at 37°C. After blocking the wells with 1% BSA in TSM (100 ml/ml; 30 minutes at 37°C), wells were coated O/N at 4°C with ICAM-1Fc or ICAM-3Fc containing supernatants at the indicated concentrations, from stable CHO-K1 transfectants generated by co-transfection of the ICAM-1 IgG1Fc, ICAM-3 IgG1Fc or CD14Fc (Fawcett et al., 1992) plasmids (20 mg), with the pEE14 plasmid (5 mg), as described (van Kooyk et al., 1999). Cells (4x10⁶/ml/well) were labeled in PBS with Calcein-A (25 mg/ml cells; Molecular probes, Eugene, OR) for 30 minutes at 37°C and incubated on ICAM coated plates for 45 minutes at 37°C, in the presence or absence of the indicated mAb. Non-adherent cells were removed by three washes with 37°C wash buffer (TSM + 0.5% SDS). Fluorescence was quantified using the Cytofluorimeter (Bio-Rad, Richmond, CA). Results are expressed as the mean percentage of adhesion of triplicate wells.

Immunofluorescence analysis

Cells (0.1x10⁶) were incubated with appropriate dilutions of mAb (10 mg/ml; 25 ml/well) in PBS containing 0.5% bovine serum albumin and 0.01% Na₂CO₃ (PBA), for 30 minutes at 4°C. After one wash with cold PBA, cells were incubated with FITC-labeled goat anti-mouse secondary antibodies (Zymed, San Francisco, CA; 1:50 dilution in PBA), for 30 minutes at 4°C. Fluorescence was measured using a FACScan® (Becton and Dickinson & Co., Oxnard, CA) after another PBA wash.

Confocal microscopy

As described for the adhesion assay, 96-well (flat bottom) plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with goat anti-human Fc antibodies (60 minutes at 37°C), 1% BSA in TSM (30 minutes at 37°C), followed by ICAM-1Fc, ICAM-3Fc or CD14Fc containing supernatants at the indicated concentrations for 1 hour at 37°C. Freshly isolated resting T cells (4x10⁶/well) were incubated on ICAM coated plates for 45 minutes at 37°C. Cells were fixed with 0.5% paraformaldehyde for 15 minutes. Fixed cells were stained with TS24 mAb (10 mg/ml) for 30 minutes at 4°C, followed by incubation with FITC-labeled goat anti-mouse secondary antibodies (Zymed, San Francisco, CA; 1:50 dilution in PBA), for 30 minutes at 4°C. Cells were attached to poly-L-lysine coated glass slides, after which cell surface distribution of LFA-1 was determined by confocal laser scanning microscopy (CLSM) at 488 nm with a krypton/argon Laser (Bio-Rad, Hercules, CA). The same instrument settings were used throughout the experiments.

Co-stimulation assay

96-well (flat bottom) plates (Maxisorb, Nunc, Roskilde, Denmark)
were coated with suboptimal concentrations of anti-CD3 antibodies (T3b, 30 ng/ml; 50 ml/well, 1 hour 37°C), followed by goat anti-human Fc antibodies (Jackson Immunoresearch Laboratories, Inc., Westgrove, PA; 4 mg/ml, 50 ml/well, 1 hour 37°C), 1% BSA (100 ml/well, 30 minutes, 37°C) and ICAM-1Fc proteins (200 ng/well; 50 ml/well, 1 hour at 37°C). Resting T cells were added (1×10^5 cells/well in RPMI 1640 (Gibco) + 10% FCS) and cultured for three days. On day 3 cells were pulsed for 16 hours with [3H]thymidine (1.52 TBq/mmol, 0.5 mCi/well, Amersham, Buckinghamshire, UK), to measure ICAM-1 dependent proliferation. Cells were cultured in the presence of the indicated function blocking antibodies at a concentration of 10 mg/ml.

RESULTS

ICAM-3 is unable to support LFA-1-mediated adhesion of resting T cells

To determine the role of ICAM-3 in adhesion of T lymphocytes, we compared the capacity of LFA-1 expressing resting T cells with activated T cells to bind to ICAM-1Fc or ICAM-3Fc proteins (Fig. 1). As resting T cells, PBL and the Peer T cell line were used, whereas the HSB-2 T cell line is more activated, as the cells grow in clusters due to the active

Fig. 1. Adhesion of resting and activated T lymphocytes to ICAM-1Fc and ICAM-3Fc. Calcein labeled activated HSB-2 T cells (A,B), resting T lymphocytes (C,D), Peer T cells (E,F), or LFA-1 transfected K562 cells (G,H) were incubated on plates coated with anti-human Fc mAb and ICAM-1Fc (A,C,E,G) or ICAM-3Fc (B,D,F,H) proteins at concentrations ranging from 25-500 ng/ml, for 45 minutes at 37°C, in the absence (diamonds) or presence of the phorbolester PMA (50 nM) (squares), or the LFA-1 activating mAb KIM185 (10 mg/ml) (triangles). After washing away non-bound cells, fluorescence was quantified. Results are expressed as the mean % of LFA-1/ICAM-1 specific adhesion, of triplicate wells. Specific adhesion: percentage of bound cells minus percentage of cells bound in the presence of a LFA-1 blocking antibody (NKI-L15). Data are representative of three experiments.
state of the integrins. Maximal adhesion of activated HSB-2 T cells to ICAM-1Fc could be observed at all ICAM-1Fc concentrations (25-500 ng/ml), without prior activation of LFA-1 (Fig. 1A), indicating that LFA-1 is quite active on HSB-2 T cells. In contrast HSB-2 T cells did not spontaneously bind ICAM-3. Upon further activation of LFA-1 with the activating antibody KIM185, HSB-2 T cells bound to ICAM-3 Fc (50%; Fig. 1B), although to a lesser extent than to ICAM-1Fc proteins (70%), since a 12-fold higher concentration of ICAM-3Fc was required for maximal adhesion. When adhesion of resting T cells was examined, they were found to adhere less well to ICAM-1Fc when compared to activated T cells (Fig. 1C), since adhesion required activation of LFA-1, and higher ICAM-1Fc concentrations (200 ng/ml) were needed for maximal adhesion (60%). In contrast, even upon activation of LFA-1, resting T cells could not adhere to ICAM-3Fc (<8%), at all concentrations tested (Fig. 1D). Similarly resting T cell lines, such as Peer T cells but also LFA-1 expressing transfectants such as K562-LFA-1 did not bind to any concentration of ICAM-3Fc coated (Fig. 1F,H), while LFA-1 expressed by these cells was capable to bind ICAM-1 (Fig. 1E,G). The fact that resting T cells bind less well ICAM-1Fc or ICAM-3Fc than activated T cells was not due to the fact that they express lower levels of LFA-1 on the cell surface (Table 1). These results clearly indicate that while activated T cells bind to both ICAM-1 and ICAM-3, resting T cells only bind to ICAM-1 and fail to bind ICAM-3, even after strong activation of LFA-1 by an activating antibody.

**Low-affinity interaction of LFA-1 with recombinant ICAM-3 enhances LFA-1 mediated adhesion to ICAM-1**

We next investigated whether low-affinity interactions of LFA-1 with ICAM-3 could regulate LFA-1-mediated adhesion to the high affinity LFA-1 ligand ICAM-1. Therefore, we analyzed adhesion of resting T cells, such as Peer T cells, to wells coated with a combination of ICAM-1Fc and ICAM-3Fc proteins, or wells coated with only ICAM-1Fc or ICAM-3Fc. Fig. 2A shows that although coated ICAM-3Fc was unable to support adhesion of Peer T cells, interaction of LFA-1 with immobilized ICAM-3Fc enhanced LFA-1-mediated adhesion of Peer T cells to ICAM-1Fc upon activation of LFA-1 with the activating antibodies MEM-48 or KIM185. The observed ICAM-3 dependent increase in adhesion was specific, since coating of a combination of ICAM-1Fc with CD14Fc on the plate did not increase LFA-1-mediated ICAM-1 adhesion (data not shown). To exclude the involvement of other β2 integrins or ICAM-3 signaling on Peer T cells, we used K562-LFA-1 transfectants that only expressed LFA-1 and no ICAM-3. Fig. 2B again demonstrates that interaction of LFA-1 with ICAM-3Fc only did not lead to any cell binding. However, when ICAM-3Fc was immobilized together with ICAM-1Fc, adhesion was specifically enhanced to ICAM-1Fc (30% increase) when stimulated with KIM185 or MEM-48. PMA did not induce any adhesion of K562-LFA-1 or Peer T cells since LFA-1 expressed in these cells is not responsive to PMA (Lub et al., 1997b).

Only little amounts of coated ICAM-3Fc were required to enhance LFA-1-mediated adhesion to ICAM-1Fc, since maximal increase of adhesion was already observed at an ICAM-3Fc concentration of 50 ng/ml (Fig. 3A). No increase of adhesion was observed in the presence of blocking
Role of ICAM-3 in LFA-1/ICAM-1 adhesion and signaling

Antibodies directed against ICAM-3, confirming that the observed increase of adhesion to ICAM-1 was ICAM-3 dependent (Fig. 3B). Together, these data show that low-affinity interactions of LFA-1 with ICAM-3 can regulate LFA-1/ICAM-1 binding in resting T cells.

Enhanced clustering of LFA-1 upon low-affinity interaction with recombinant ICAM-3

Since clustering of LFA-1 on the cell surface has been shown to enhance LFA-1-mediated adhesion to ICAM-1 (Lub et al., 1995; van Kooyk et al., 1994) we investigated whether low-affinity LFA-1/ICAM-3 interactions enhanced binding to ICAM-1 through reorganizing LFA-1 on the cell surface of resting T cells. Using confocal laser scan microscopy we observed that the surface membrane distribution of LFA-1 on resting T cells, stained with the non-activating mAb TS2/4, is dramatically altered upon ICAM-3 binding (Fig. 4). We found that LFA-1 is homogeneous distributed on resting T cells upon interaction with uncoated plates (Fig. 4A) or coated with ICAM-1Fc or ICAM-3Fc alone (Fig. 4B,C). In contrast, when resting T cells came in contact with both immobilized ICAM-1Fc and ICAM-3Fc (Fig. 4E,F), the organization of LFA-1 on the cell surface is dramatically altered into a more clustered distribution. The number of LFA-1 molecules was not increased since surface expression experiments (FACS analysis) resulted in equal staining of LFA-1 in all experimental settings (Table 2), nor was LFA-1 activated as determined by activation-epitope-dependent mAb NKI-L16 and M24 (Table 2). In addition, binding of PBLs to ICAM-1, ICAM-3 or ICAM-1+ICAM-3 is not due to enrichment for any T cell subsets such as CD4, CD8, CD45RA, or CD45RO as shown in Table 2. Furthermore, the induced clustering of LFA-1 was ICAM-3 specific, since coating of CD14Fc together with ICAM-1Fc did not induce a clustering of LFA-1 on the cell membrane, as the LFA-1 distribution was similar as on T cells that came in contact with non-coated wells (Fig. 4D). This indicates that the low-affinity interaction of LFA-1 with ICAM-3 and ICAM-1 primes LFA-1 to form clusters. Further activation of LFA-1 by activating antibodies can than enhance the adhesion of T cells to ICAM-1.

Table 2. Expression patterns of PBL after adhesion to ICAM-1 and/or ICAM-3 substrate

<table>
<thead>
<tr>
<th>Coating</th>
<th>Control</th>
<th>SPV-L7</th>
<th>NKI-L16</th>
<th>M24</th>
<th>M24 Mn²⁺</th>
<th>CD4</th>
<th>CD8</th>
<th>CD45RO</th>
<th>CD45RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>2 (3)</td>
<td>274 (99)</td>
<td>88 (99)</td>
<td>23 (10)</td>
<td>124 (98)</td>
<td>119 (45)</td>
<td>372 (36)</td>
<td>110 (73)</td>
<td>107 (78)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>3 (1)</td>
<td>258 (99)</td>
<td>60 (96)</td>
<td>7 (2)</td>
<td>103 (98)</td>
<td>120 (51)</td>
<td>411 (40)</td>
<td>116 (73)</td>
<td>100 (76)</td>
</tr>
<tr>
<td>ICAM-3</td>
<td>3 (3)</td>
<td>278 (99)</td>
<td>66 (94)</td>
<td>8 (3)</td>
<td>84 (96)</td>
<td>103 (60)</td>
<td>410 (40)</td>
<td>118 (74)</td>
<td>97 (69)</td>
</tr>
<tr>
<td>ICAM-1+ICAM-3</td>
<td>3 (3)</td>
<td>282 (99)</td>
<td>70 (96)</td>
<td>7 (8)</td>
<td>92 (98)</td>
<td>117 (51)</td>
<td>403 (40)</td>
<td>112 (71)</td>
<td>103 (72)</td>
</tr>
</tbody>
</table>

*Mean fluorescence and (percentage positive cells).

PBL were allowed to adhere for 45 minutes at 37°C to wells coated with medium, 40 ng/ml ICAM-1, 50 ng/ml ICAM-3 or 40 ng/ml ICAM-1 and 50 ng/ml ICAM-3.

Antibodies used: SPV-L7, anti-CD11a; NKI-L16 and M24 are LFA-1 activation-dependent-epitope antibodies; M24 with 2mM Mn²⁺; RIV-6 for anti-CD4; wt82 for anti-CD8; anti-CD45RO and anti-CD45RA.
LFA-1/ICAM-3 interactions are critical for ICAM-1 induced T cell proliferation

Since these results indicate that low-affinity interactions of LFA-1 with ICAM-3 enhance LFA-1/ICAM-1 dependent adhesion, we subsequently investigated the role of ICAM-3 in LFA-1/ICAM-1 dependent T cell proliferation. To this end we analyzed co-stimulation of T cells induced by adding T cells by coated ICAM-1Fc proteins and suboptimal amounts of anti-CD3 antibodies (Fig. 5A). Incubation of resting T cells on these ICAM-1Fc and anti-CD3 coated plates resulted in strong proliferation, that was LFA-1 and ICAM-1 dependent, since it was prevented by addition of blocking antibodies directed against CD11a or ICAM-1. Surprisingly, LFA-1/ICAM-1
induction of T cell proliferation, cytotoxicity, and expression of signals transmitted by LFA-1 upon ICAM-1 binding result in the ICAM-1 cell-cell interaction will be induced (4). Ultimately, signals enhance the affinity of LFA-1 for ICAM-1 (3), stable LFA-1 redistribution/clustering of LFA-1 (2), to prime LFA-1 for activation. When upon anti-CD3 triggering or antigen recognition intracellular signaling between T and/or B cells. Depicted is the interaction of an effector cell with a target cell that expresses both ICAM-1 and ICAM-3. Low-affinity interaction of LFA-1 on the T cell with ICAM-3 on the target cell (1), induces adhesion of LFA-1 to ICAM-3 molecules (Cabanas and Hogg, 1993; Buckley et al., 1997), it is hard to envisage how LFA-1 molecules can maintain a conformation induced by weak interaction with ICAM-3 once this ligand dissociates from LFA-1. Alternatively, upon engagement of LFA-1 by ICAM-3, LFA-1 may transmit signals into the cell that enhance LFA-1 function. Upon ligand binding, β1 and β3 integrins organize into structures called focal adhesions that strengthen integrin adhesion by linking them to the cytoskeleton. These focal adhesions contain a variety of cytoskeletal proteins and signaling molecules (Burridge and Fath, 1989; Craig and Johnson, 1996; Clark and Brugge, 1995). Whether β2 integrins form similar focal adhesions is not well established. However, it has been shown that the β2 cytoplasmic tail can link to the cytoskeletal proteins actin and filamin (Pavalko and LaRoche, 1993). It is tempting to speculate that upon engagement of LFA-1 by ICAM-3, proteins involved in β2 mediated signal transduction associate with the cytoplasmic domains of LFA-1, thereby facilitating stable interaction of LFA-1 with ICAM-1.

Certainly, the expression levels of LFA-1 are not different on activated versus resting T cells. However the distribution of LFA-1 on activated T cells such as HSB-2 is extremely different than that on resting T cells (data not shown). We earlier reported that the clustered distribution of LFA-1 on activated T cells, versus the homogeneous distribution of LFA-1 on the surface of resting PBL reflects the adhesive state of LFA-1 (van Kooyk et al., 1994; Figdor et al., 1990). Clustering of LFA-1 seems to regulate LFA-1 activation dynamically since it has been reported by various groups that avidity alterations in LFA-1 enhance LFA-1/ICAM-1 adhesion. It has been demonstrated that during activation of resting T cells in culture, LFA-1 organizes into clusters on the cell surface and facilitates LFA-1/ICAM-1 interactions (van Kooyk et al., 1991, 1994; Kucik et al., 1996). Similarly, also disconnection of LFA-1 from the cytoskeleton network by agents that disrupt the actin cytoskeleton, such as cytochalasin D, or by deletion of the cytoplasmic tails, has been shown to result in a constitutively active LFA-1 receptor, that is redistributed into

**DISCUSSION**

ICAM-3 has been described as an adhesive and signaling counter receptor for LFA-1, although the function of ICAM-3 as an adhesion ligand for LFA-1 remains poorly defined. We therefore investigated the role of ICAM-3 on LFA-1/ICAM-1 dependent cell adhesion in a purified protein adhesion assay as well as in cell-cell interactions that take place within the immune system such as T cell proliferation.

We found that purified ICAM-3 is unable to support adhesion of resting T lymphocytes, indicating that ICAM-3 does not directly contribute to the LFA-1-mediated adhesion of these cells. However, low-affinity interaction of LFA-1 with recombinant ICAM-3 or cell-bound ICAM-3 enhances LFA-1 dependent adhesion to ICAM-1 and proliferation. By confocal microscopy studies we demonstrated that ICAM-3 in combination with ICAM-1 enhances clustering of LFA-1 on the cell surface of resting T cells, thereby enhancing the avidity of LFA-1/ICAM-1 interactions. We demonstrated that low-affinity LFA-1/ICAM-3 dependent intercellular interactions are critical for co-stimulation of resting T cells induced by recombinant ICAM-1. This indicates that interaction of the LFA-1/ICAM-3 binding does not directly contribute to intercellular adhesion of T cells but rather induces subtle conformational changes in LFA-1, such as redistribution of LFA-1 on the cell surface, that allow optimal adhesion to ICAM-1 and subsequent signaling.

Low-affinity LFA-1/ICAM-3 interactions enhance LFA-1 function but are not sufficient to induce adhesion of LFA-1 to ICAM-1, since enhancement of adhesion is only observed upon activation of LFA-1 with, LFA-1 activating antibodies, anti-CD3, or antigen (Figs 2, 5, 6). It is possible that binding of ICAM-3 to LFA-1 induces a ligand-dependent conformational change in LFA-1 that facilitates its subsequent interaction with ICAM-1. Although it has been shown that fixation of LFA-1 molecules in an ICAM-1 bound state facilitates subsequent interaction with other ICAM-1 or ICAM-3 molecules (Cabanas and Hogg, 1993; Buckley et al., 1997), it is hard to envisage how LFA-1 molecules can maintain a conformation induced by weak interaction with ICAM-3 once this ligand dissociates from LFA-1. Alternatively, upon engagement of LFA-1 by ICAM-3, LFA-1 may transmit signals into the cell that enhance LFA-1 function. Upon ligand binding, β1 and β3 integrins organize into structures called focal adhesions that strengthen integrin adhesion by linking them to the cytoskeleton. These focal adhesions contain a variety of cytoskeletal proteins and signaling molecules (Burridge and Fath, 1989; Craig and Johnson, 1996; Clark and Brugge, 1995). Whether β2 integrins form similar focal adhesions is not well established. However, it has been shown that the β2 cytoplasmic tail can link to the cytoskeletal proteins actin and filamin (Pavalko and LaRoche, 1993). It is tempting to speculate that upon engagement of LFA-1 by ICAM-3, proteins involved in β2 mediated signal transduction associate with the cytoplasmic domains of LFA-1, thereby facilitating stable interaction of LFA-1 with ICAM-1.

Certainly, the expression levels of LFA-1 are not different on activated versus resting T cells. However the distribution of LFA-1 on activated T cells such as HSB-2 is extremely different than that on resting T cells (data not shown). We earlier reported that the clustered distribution of LFA-1 on activated T cells, versus the homogeneous distribution of LFA-1 on the surface of resting PBL reflects the adhesive state of LFA-1 (van Kooyk et al., 1994; Figdor et al., 1990). Clustering of LFA-1 seems to regulate LFA-1 activation dynamically since it has been reported by various groups that avidity alterations in LFA-1 enhance LFA-1/ICAM-1 adhesion. It has been demonstrated that during activation of resting T cells in culture, LFA-1 organizes into clusters on the cell surface and facilitates LFA-1/ICAM-1 interactions (van Kooyk et al., 1991, 1994; Kucik et al., 1996). Similarly, also disconnection of LFA-1 from the cytoskeleton network by agents that disrupt the actin cytoskeleton, such as cytochalasin D, or by deletion of the cytoplasmic tails, has been shown to result in a constitutively active LFA-1 receptor, that is redistributed into
clusters on the cell surface (Lub et al., 1997a; van Kooyk et al., 1999). This clearly demonstrates the importance of avidity changes that stimulate adhesive properties of LFA-1. In this study we show that also the cell surface redistribution of LFA-1 can dramatically alter upon ICAM-3 ligand binding (low affinity interaction of ICAM-3 in combination with ICAM-1), and results in an increase of the avidity of LFA-1/ICAM-1 interactions. This finding gives ICAM-3 a new function being not only a low affinity adhesion ligand for LFA-1 but regulate LFA-1 avidity by altering the surface distribution of LFA-1 on resting T cells.

ICAM-3 induced LFA-1-mediated binding to ICAM-1 has been reported in the past, however, in these studies ICAM-3 adhesion was achieved by activating anti-ICAM-3 antibodies that induce T cell aggregation (Campanero et al., 1993). In these studies it is still questionable whether LFA-1/ICAM-1 interactions were the result of initial ICAM-3 binding or by ICAM-3-mediated signaling through crosslinking ICAM-3 with an antibody. Several groups have reported that antibody crosslinking of ICAM-3 induces co-stimulation, elevation of intracellular calcium levels, activation of Src family kinases, tyrosine phosphorylation of intracellular proteins, and morphological changes in T cells (Juan et al., 1994; Arroyo et al., 1994; Hernandez-Caselles et al., 1993; Campanero et al., 1993; Cid et al., 1994; Delpozo et al., 1994). It remains possible that low-affinity LFA-1/ICAM-3 interactions are sufficient to elicit ICAM-3 dependent signal transduction in lymphocytes. So far ICAM-3 dependent signaling has only been studied upon crosslinking with anti-ICAM-3 antibodies. Whether binding of β2 integrins to ICAM-3 result in ICAM-3 dependent signal transduction remains to be seen. We here show that ICAM-3 induced LFA-1/ICAM-1 binding occurs equally well upon initial interaction of LFA-1 to ICAM-3 expressed on cells or presented as purified protein (ICAM-3Fc), clearly demonstrating that this interaction is independent from ICAM-3 induced signaling.

Our results indicate that ICAM-3 plays a pivotal role in the regulation of various leukocyte intercellular interactions. Despite the low affinity of LFA-1 for ICAM-3 the high expression of ICAM-3 on resting leukocytes, compared to ICAM-1 or ICAM-2, makes it an ideal initial counter-receptor for LFA-1. Accordingly, it has been demonstrated that adhesion of resting T lymphocytes to LFA-1 occurs primarily via ICAM-3 (de Fougerolles and Springer, 1992). In a second step, the initial interaction of ICAM-3 with LFA-1 on the opposite cell can affect the redistribution of LFA-1 into clusters, facilitating LFA-1-mediated adhesion to ICAM-1, which establishes a more stable cell-cell interaction. This putative role of ICAM-3 can be essential to initiate T cell proliferation after TCR engagement, mixed lymphocyte reactions, in lysis of target cells by NK cells, and T-B cell interactions.

In summary, our results for the first time show that physiological low-affinity interactions between LFA-1 and ICAM-3 strengthen LFA-1 dependent ICAM-1 adhesion. Based on our results we propose a novel model for the interaction of resting T cells with target cells such as T or B cells (Fig. 6). In this model, low-affinity interactions of LFA-1 expressed on the T cell occur with ICAM-3 and ICAM-1 on the counter cell, which is not measurable in adhesion assays. This low affinity ICAM-3 binding redistributes LFA-1 on the cell surface by reorganization of LFA-1 into clusters, making it ready to bind ICAM-1. When intracellular signals arrive the clustered LFA-1, such as via or through anti-CD3 triggering or antigen recognition, these signals activate LFA-1. The clustered and activated LFA-1 can then bind its high affinity ligand ICAM-1, allowing the T cell to bind to the counter cell and to enable proliferation and proper delivery of the cytolytic hit. Since ICAM-3 is restricted to cells of hematopoietic origin, it is tempting to speculate that the main function of ICAM-3 in the interactions between T and B cells is to enhance the adhesive interaction of LFA-1 with ICAM-1 through redistribution of LFA-1.

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REFERENCES


