**Brief Definitive Report**

**Lymphocyte Function-associated Antigen 1 Dominates Very Late Antigen 4 in Binding of Activated T Cells to Endothelium**

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**Summary**

Lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 (LFA-1/ICAM-1)- and very late antigen 4/vascular cell adhesion molecule 1 (VLA-4/VCAM-1)-mediated adhesion of T lymphocytes to endothelial cells (EC) can be regulated by increased expression of ICAM-1 and VCAM-1 upon cytokine treatment of EC, or by activation of the integrin molecules LFA-1 and VLA-4 on T cells. Here, we provide evidence that preferential usage of LFA-1 over VLA-4 is yet another mechanism to control T cell adhesion. We observed that binding of activated T lymphocytes, as opposed to resting T cells, to EC is essentially mediated through LFA-1 and not through VLA-4. VLA-4-mediated adhesion of T cells to EC is only found when LFA-1 is not expressed or not functional, as observed for several T cell leukemia cell lines. These results suggest that LFA-1-mediated adhesion dominates and may downregulate VLA-4-mediated adhesion through an unidentified mechanism.

**Materials and Methods**

**Reagents and Antibodies.** The mAbs used in this study were SPV-L7 directed against the α chain of LFA-1 (CD11a); 15), NKI-L16 directed against a Ca²⁺-dependent epitope on LFA-1 (CD11a*; 16), CLB LFA-1/1 reactive with the β2 chain (CD18; 16), F10.2 (anti-ICAM-1, CD54); HP2/1 (anti-VLA-4, CD49d; 17); TS2/16 directed against β1 (CD29; 18); 4B9 (anti-VCAM-1); and ENA-1 (anti-endothelial leukocyte adhesion molecule 1 [ELAM-1]). Reagents used were human TNF-α (100 U/ml; supernatant of cDNA-transfected COS cells), phorbol ester PMA (50 ng/ml; Sigma Chemical Co., St. Louis, MO), and fibronectin (FN) (20 µg/ml; Sigma Chemical Co.).

**Cells.** The T cell clone JS136 used in this study was cultured as described previously (9). The LFA-1-deficient T cell clone (LAD 6.6) was raised from PBL of a patient suffering from the leukocyte adhesion deficiency (LAD) syndrome (19) and was cultured under the same conditions as T cell clone JS136. The human leukemic T cell lines Jurkat and CEM were cultured in Iscove’s medium containing 5% FCS. A homogenous population of highly purified T lymphocytes was isolated by centrifugal elutriation (9), and cultured in Iscove’s medium containing 5% FCS and IL-2 (100 U/ml; Cetus Corp., Emeryville, CA) for 24 h. L-ICAM-1 cells were obtained by transfection of ICAM-1 cDNA into mouse fibroblast L cells. ICAM-1 expression was high (95%; mean, 45) and remained throughout culture of the cells in the presence of Hygromycin B (200 µg/ml; Schering Research, Bloomfield, NJ). Human EC were isolated from umbilical vein by collagenase digestion, and cultured...
as described previously (20). Cells from passages one to three were used for adhesion experiments.

Clustering Assay. Binding of T cells to L-ICAM-1 cells was determined by means of double fluorescence. Cells (10⁶/ml) were stained with the green dye sulfofluorescein diacetate (SFDA; Molecular Probes, Junction City, OR) at a concentration of 5 μg/ml or with the red dye Hydroethidine (HE; Polyscience Inc., War- rington, PA) at a concentration of 3 ng/ml, as described previously (9). 10⁶ red-labeled cells and 10⁶ green-labeled cells were incubated at 37°C for different periods of time in Iscove’s medium containing 0.5% BSA, and stimulated with PMA (50 ng/ml). Subsequently, cells were fixed with 0.5% (wt/vol) paraformaldehyde, and heterotypic conjugates were measured by FACScan® analysis (Becton Dickinson & Co., Mountain View, CA). Data are representative of four experiments.

Adhesion Assay. Human EC were seeded at 2 × 10⁵ cells/ml in FN-coated (2 μg/ml) 96-well plates and were stimulated for 24 h with human TNF-α (100 U/ml). Adhesion experiments were performed as described previously (20). Briefly, ⁵¹Cr-labeled T cells were allowed to bind at 37°C for 30 min. The number of adherent T cells was quantified in a gamma counter. Results are expressed as the mean percentage of cells binding from triplicate wells. For inhibition studies, cells were preincubated (30 min, 4°C) with 1:100 ascites dilution or 10 μg/ml purified mAb. Data are representative for four experiments. For adhesion experiments to VCAM-1, purified soluble VCAM-1 (21) (0.8 μg/ml) was coated for 16 h at 4°C. Subsequently, wells were coated by 1% (wt/vol) BSA for 1 h at 37°C. T cell adhesion was performed under the same conditions as described for EC.

Immunofluorescence. Cells were incubated for 30 min at 4°C in PBS, 0.5% (wt/vol) BSA, 0.2% azide with appropriate dilutions of the mAb, followed by incubation with FITC-labeled goat (Fab’)-anti-mouse IgG antibody (GAM-FITC; Nordic, Tilburg, the Netherlands) for 30 min at 4°C. The relative fluorescence intensity was measured by FACScan® analysis (Becton Dickinson and Co.).

### Results and Discussion

The contribution of the LFA-1/ICAM-1 and VLA-4/VCAM-1 adhesion receptor pairs in T cell–EC interactions was examined by binding of resting and activated T cells to 24-h TNF-α-cultured EC, which expressed high levels of ICAM-1 and VCAM-1, and only low levels of ELAM-1 (Table 1). LFA-1+ T cells (JS136, PBL, CEM, and Jurkat) as well as LFA-1− T cells (LAD 6.6), obtained from a patient suffering the LAD syndrome (19), showed significant binding to TNF-α-stimulated EC (Fig. 1A). Interestingly, we observed that, although approximately equal numbers of the different cell types bound to EC (except resting PBL), distinct receptor pairs were used to mediate adhesion. T cell clone JS136 and IL-2-cultured lymphocytes showed LFA-1-restricted adhesion (blocked by anti-CD18 antibodies; Fig. 1B). In contrast, the LFA-1− T cell clone (LAD 6.6) and two LFA-1+ T cell lines (CEM and Jurkat) bound to TNF-α–EC exclusively through VLA-4 (Fig. 1B). Compared with the cultured T cells, binding of freshly isolated lymphocytes to TNF-α-stimulated EC was significantly lower (Fig. 1A) and was mediated both by LFA-1 and VLA-4 (Fig. 1B). Antibodies directed against ICAM-1 and VCAM-1 blocked the adhesion of the cells to the same extent as anti-LFA-1 or anti-VLA-4 antibodies, respectively (not shown). Antibodies to ELAM-1 did not block the adhesion, indicating that ELAM-1 is not involved in this process (not shown; 20). These results indicate that only resting PBL use both adhesion pathways (LFA-1/ICAM-1 and VLA-4/VCAM-1). Upon in vitro culture of PBL there is a tendency towards LFA-1-dependent/VLA-4-independent adhesion, whereas fully activated T cells, like a T cell clone (several other T cell clones were studied; not shown), exclusively use LFA-1 but not VLA-4. In con-

### Table 1. Expression of Cell Adhesion Molecules on Different T Cells and Endothelial Cells

<table>
<thead>
<tr>
<th>Relative fluorescence intensity</th>
<th>T cells</th>
<th>Cultured endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb Antigen</td>
<td>JS136</td>
<td>Ly</td>
</tr>
<tr>
<td>GAM-FITC</td>
<td>Control</td>
<td>2</td>
</tr>
<tr>
<td>SPV-L7</td>
<td>LFA-1</td>
<td>307</td>
</tr>
<tr>
<td>NIKI-L16</td>
<td>LFA-1*</td>
<td>304</td>
</tr>
<tr>
<td>HP 2/1</td>
<td>VLA-4</td>
<td>74</td>
</tr>
<tr>
<td>FI0.2</td>
<td>ICAM-1</td>
<td>40</td>
</tr>
<tr>
<td>4B9</td>
<td>VCAM-1</td>
<td>2</td>
</tr>
<tr>
<td>ENA-1</td>
<td>ELAM-1</td>
<td>3</td>
</tr>
</tbody>
</table>

Lymphocytes (Ly) were freshly isolated or cultured for 24 h with 100 U/ml IL-2 (Ly IL-2). The LFA-1+ and LFA-1− T cell clones JS136 and LAD6.6, respectively, and the T cell lines Jurkat and CEM were cultured as described in Materials and Methods. The EC were activated by culturing for 24 h in the presence of 100 U/ml TNF-α. Antigen expression was determined by immunofluorescence. One representative experiment out of four is shown.

* Ca²⁺-dependent epitope.
Contrast to this shift from LFA-1/VLA-4-mediated adhesion to only LFA-1-mediated adhesion, we observed that several leukemic T cell lines that express significant levels of LFA-1 (Table 1) bind EC exclusively through VLA-4. In all cases adhesion of T cells to TNF-α-stimulated EC could not be blocked completely by anti-LFA-1 and anti-VLA-4 antibodies (up to 60–80% of total adhesion; Fig. 1 C), indicating that other as yet undefined adhesion structures mediate the remaining 20–40% of adhesion.

The selective use of LFA-1 or VLA-4 by T cells to mediate adhesion to EC prompted us to investigate the expression of these adhesion receptors in more detail (Table 1). All T cells expressed significant levels of VLA-4, indicating that in principle all cells are capable of using VLA-4 to mediate adhesion to TNF-α-stimulated EC. As expected, LFA-1 expression is totally absent on LAD T cells (LAD 6.6), thus explaining the VLA-4-mediated binding to TNF-α-stimulated EC. However, all other T cells expressed significant levels of LFA-1 (JS136, lymphocytes, CEM, and Jurkat). LFA-1 is expressed at much higher levels on JS136 compared with PBL, CEM, and Jurkat. Interestingly, we observed that expression of the L16 activation epitope, a Ca²⁺-dependent epitope on LFA-1, which recognizes a "potentially active" form of LFA-1 (9), is absent on the LFA-1⁺ CEM and Jurkat T cells, whereas expression is low on resting lymphocytes (9) and high on IL-2-cultured lymphocytes and on T cell clone JS136. Earlier work indicated that expression of the L16 epitope is a prerequisite for LFA-1 to mediate cell adhesion (9, 14). This finding suggests that JS136 and IL-2-cultured PBL express a form of LFA-1 that can readily be activated to high-avidity ligand binding, whereas LFA-1 expressed by CEM and Jurkat cells lacks L16 expression and can therefore not reach its activated state, thus explaining their LFA-1-independent, VLA-4-mediated adhesion to TNF-α-stimulated EC. The dull expression of the L16 epitope on resting lymphocytes correlates with the observation that the interaction of resting PBL is only partially mediated by LFA-1 since only a small number of the expressed LFA-1 molecules can become activated.

To determine the functional activity of the LFA-1 adhesion receptors expressed on these T cells, we examined the capacity of these T cells to bind L cell transfectants expressing ICAM-1 (Fig. 2). It is known that LFA-1-mediated adhesion can be induced by the addition of PMA to T cells, resulting in high-avidity ligand binding (22). The LFA-1⁻ T cells (LAD 6.6), which were used as a control, could not bind to L-ICAM-1 (Fig. 2). As expected, binding of L16⁺ JS136 T cells and lymphocytes to L-ICAM-1 cells was induced upon addition of PMA, and could be blocked completely to background levels by anti-CD18 or anti-CD54 antibodies (not shown). In contrast, addition of PMA to the LFA-1⁺ T cells (CEM and Jurkat) did not result in activation of LFA-1, because no binding to L-ICAM-1 cells was observed. To exclude the possibility that this was caused by the relatively low expression of LFA-1 on these cells (compared with JS136), we determined the binding capacity of PBL that were briefly cultured in IL-2 to express approximately similar levels of LFA-1 as Jurkat and CEM. In contrast to the latter two cell lines,
Figure 2. Clustering of T cells with L-ICAM-1 cells. Cells were differentially labeled with fluorescent dyes. Clustering was induced with (▲) or without (■) the addition of PMA (50 ng/ml) for 45 min at 37°C. Values represent means of duplicate wells. Clustering of T cells with mock-transfected control cells was always <10%. One experiment out of three is shown.

Figure 3. Adhesion of T cells to VCAM-1. T cells were stimulated with (▲) or without (■) anti-β1 antibody TS2/16 (10 μg/ml) for 10 min at 4°C. Subsequently, adhesion to purified VCAM-1 was performed for 30 min at 37°C. TS2/16-induced adhesion was blocked with an inhibitory anti-VLA-4 (HP2/1, 10 μg/ml) antibody (■). Adhesion to BSA was always <5%. One experiment out of two is shown.

Figure 4. Contribution of LFA-1 and VLA-4 to TS2/16-induced JS136 T cells binding to TNF-α-EC. 51Cr-labeled T cells were allowed to adhere for 30 min at 37°C to 24-h TNF-α-stimulated EC. Adhesion was induced by the addition of anti-β1 antibody TS2/16 (10 μg/ml) or PMA (50 ng/ml). Basal adhesion of T cells was 50% adhesion, and was induced by TS2/16 and PMA up to 60%. Values are percentages of inhibition by the addition of anti-CD18 (■) or anti-VLA-4 (■) (10 μg/ml). One experiment out of three is shown.
or Jurkat T cells did not alter the VLA-4-dependent adhesion to EC into a LFA-1-dependent adhesion, providing further evidence that their LFA-1 molecules are not functional (not shown).

It should be noted that binding of T cells to isolated ligands (ICAM-1, VCAM-1, and FN) or ligands expressed by transfected L cells is low unless the T cells are activated by PMA or other stimuli, inducing a high affinity state of the integrin receptor (9, 13, 25). Nevertheless, we consistently observed strong binding of the cells used in this study to EC via these ligands (ICAM-1 and VCAM-1). This suggests that other interactions precede engagement of LFA-1 or VLA-4, as has been demonstrated for ELAM-1 (26). It can be excluded that E-selectin and L-selectin are involved in this process (26, 27). Activated T cells lack L-selectin expression, whereas E-selectin expression is low on EC after prolonged (24-h) exposure to TNF-α (Table 1). This indicates that other, undefined molecules expressed by these T cells may induce high affinity binding of VLA-4 or LFA-1, upon binding of TNF-α–stimulated EC. One possible candidate is CD31, which has recently been described to stimulate β1- and β2-mediated adhesion of T cell subsets to VCAM-1 and ICAM-1 (28). CD31 seems to preferentially stimulate β1-mediated adhesion, whereas in our study, β2-mediated adhesion seems to dominate β1-mediated adhesion, suggesting that also other molecules may be involved.

Since transendothelial migration of T cells mainly involves the LFA-1/ICAM-1 interaction (29), and not the VLA-4/VCAM-1 interaction, the absence of the L16 epitope on LFA-1 can have serious effects on the transendothelial migration capacity of LFA-1†L16− T cells. Indeed, it has been reported that migration of LFA-1− (LAD) T cells through EC is severely affected by the absence of LFA-1 (30). We therefore assume that LFA-1+ T cells, which lack the L16 epitope, show binding to EC using VLA-4, but migrate poorly through EC. In contrast, LFA-1+L16+ T cells will readily bind EC, and migrate through EC using high affinity LFA-1. This may provide the immune system with a mechanism by which preferentially activated LFA-1†L16+ T cells will be capable of migrating into tissues and actively participating in the effector phase of an inflammatory/immune response.

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References


