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 moleculer 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.

Adhesion of T lymphocytes to endothelium, lining the blood vessels, is a crucial step in immune surveillance. It allows T lymphocytes to recirculate and migrate into sites of inflammation (1). Different adhesion receptors have been described to be involved in this process (2, 3). The integrins LFA-1 and very late antigen 4 (VLA-4), which are both expressed on T cells, have been reported to mediate binding to endothelial cells (EC) (4–7). One mechanism to regulate adhesion of T lymphocytes to endothelial cells involves activation of EC by inflammatory cytokines such as TNF-α, which results in a rapid increase in the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), ligands of LFA-1 and VLA-4, respectively (3, 8). Recently, the activation state of these integrin molecules expressed by T cells also has been described to play an important role in the regulation of the adhesion function of these cells (9–13). Here we demonstrate that yet another mechanism can regulate cell adhesion. By using an antibody (NKI-L16) that recognizes an activation epitope (termed L16) on LFA-1 (9, 14), we observed that the functional state of the LFA-1 molecule, as reported by this antibody, determines if LFA-1 or VLA-4 is exploited by T cells to bind EC. The results suggest that, only when LFA-1 is not capable to mediate adhesion, VLA-4 is used by T lymphocytes to bind EC, pointing to a selective use of these adhesion receptors by T cells.

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 Ca^{2+}-dependent epitope on LFA-1α (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 rTNF-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 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

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Volume 177 January 1993 185–190

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 (10^6/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., Warrington, PA) at a concentration of 3 ng/ml, as described previously (9). 10^5 red-labeled cells and 10^5 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 2×10^5 cells/ml in FN-coated (2 μg/ml) 96-well plates and were stimulated for 24 h with human rTNF-α (100 U/ml). Adhesion experiments were performed as described previously (20). Briefly, ^{51}Cr-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 $(Fab')_2$ 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. 1 *A*). 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. 1 *B*). 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. 1 *B*). Compared with the cultured T cells, binding of freshly isolated lymphocytes to TNF-α-stimulated EC was significantly lower (Fig. 1 *A*) and was mediated both by LFA-1 and VLA-4 (Fig. 1 *B*). 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*

		Relative fluorescence intensity							
		T cells						Cultured endothelial cells	
mAb	Antigen	JS136	Ly	Ly IL-2	Jurkat	CEM	LAD 6.6	Medium	24 h TNF-α
GAM-FITC	Control	2	1	1	2	3	3	2	4
SPV-L7	LFA-1	307	44	42	40	31	4	1	1
NKI-L16	LFA-1*	304	19	31	3	5	3	2	3
HP 2/1	VLA-4	74	41	30	23	46	130	1	1
F10.2	ICAM-1	40	5	21	16	14	29	44	394
4B9	VCAM-1	2	2	3	3	2	3	4	73
ENA-1	ELAM-1	3	6	5	4	4	4	6	14

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.

* Ca^{2+}-dependent epitope.

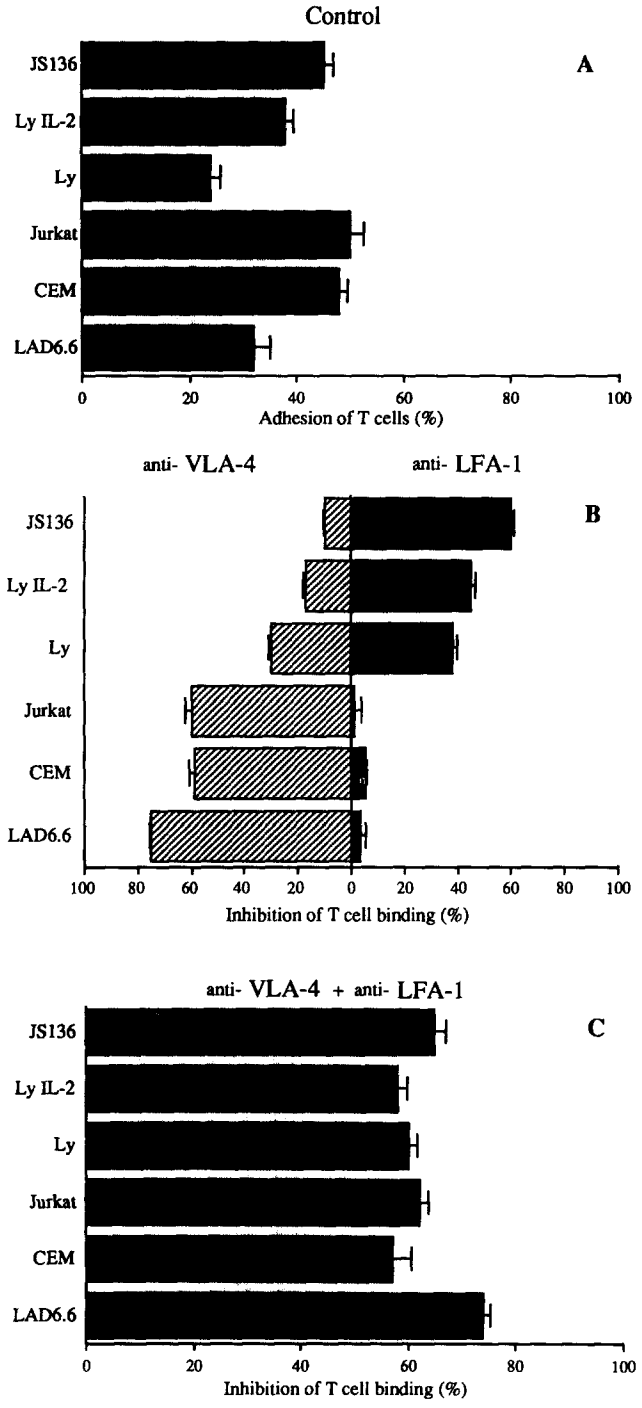

Figure 1. Adhesion of T cells to 24-h TNF-α-stimulated endothelial cells. LFA-1$^+$ T cells: T cell clone JS136, freshly isolated lymphocytes (Ly), lymphocytes cultured for 24 h with 100 U/ml IL-2 (Ly IL-2), and T cell lines Jurkat and CEM. LFA-1$^-$ T cells: T cell clone LAD 6.6. (*A*) T cells were ^{51}Cr labeled and were allowed to adhere for 30 min at 37°C to 24-h TNF-α (100 U/ml)-stimulated endothelial cells. (*B*) Percentage of inhibition of T cells to TNF-α-stimulated EC by anti-CD18 antibodies (■) or anti-VLA-4 antibodies (▨), showing the contribution of LFA-1, compared with VLA-4, in binding to EC. (*C*) Inhibition of a combination of anti-CD18 and anti-VLA-4 antibodies. The SE bars represent three independent tests within the experiment. One representative experiment out of four is shown.

trast 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 Ca^{2+}-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$^+$ L16$^-$ 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,

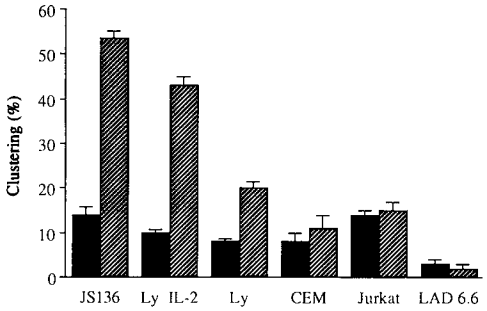

Figure 2. Clustering of T cells with L-ICAM-1 cells. Cells were differentially labeled with fluorescent dyes. Clustering was induced with (▨) or without (■) the addition of PMA (50 ng/ml) for 45 min at 37°C. Values represent means of duplicate wells. Clustering of T cells with mock-transfected control cells was always <10%. One experiment out of three is shown.

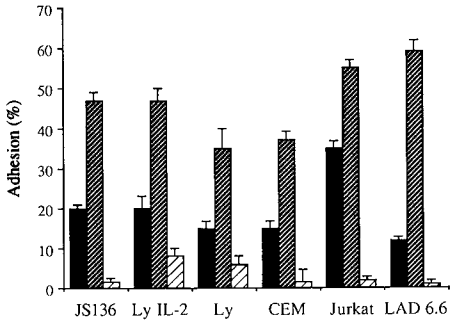

Figure 3. Adhesion of T cells to VCAM-1. T cells were stimulated with (▨) or without (■) anti-β1 antibody TS2/16 (10 μg/ml) for 10 min at 4°C. Subsequently, adhesion to purified VCAM-1 was performed for 30 min at 37°C. TS2/16-induced adhesion was blocked with an inhibitory anti-VLA-4 (HP2/1, 10 μg/ml)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

these L16$^+$ lymphocytes could readily be induced by PMA to bind L-ICAM-1, demonstrating that the level of LFA-1 expression is not limiting. Also, other stimuli known to activate LFA-1 (anti-CD2, -CD3, or the addition of Mn^{2+}; 9, 11, 23) were incapable of inducing the activated state of the LFA-1 receptor on Jurkat and CEM T cells (not shown).

The results from this study demonstrate that the selective use of adhesion receptors in adhesion and migration of T cells into sites of inflammation is not only regulated by an increased expression of the adhesion receptors' ligands ICAM-1 and VCAM-1 by inflammatory cytokines, such as TNF-α, but is also dependent on the activated state of the adhesion receptor itself, expressed on the T cell. Expression of the Ca^{2+}-dependent L16 epitope on LFA-1 determines if the LFA-1 adhesion receptor is in a "potentially active" state. Because L16 is a Ca^{2+}-dependent epitope, Ca^{2+} cations may play an important role in the formation of this conformation of LFA-1 (9). If LFA-1 expressed on T cells lacks the L16 epitope, it cannot be triggered to create a high affinity ligand binding form. To circumvent this defect these cells may use the VLA-4 receptor VCAM-1 interaction as an alternative adhesion route, which is used by the LFA-1$^-$ T cells, as well as the Jurkat and CEM T cells. On the other hand, if LFA-1 on T cells expresses the L16 epitope, LFA-1 mediates adhesion to endothelium, without any contribution of VLA-4 (Fig. 1). It is tempting to speculate that when the LFA-1/ICAM-1 interaction takes place, the VLA-4/VCAM-1 contribution in T cell/TNF-α–EC interaction is downregulated through an unknown mechanism. Work is in progress to test this hypothesis.

To exclude the possibility that VLA-4 expressed on JS136 is defective, and therefore not able to mediate adhesion to its ligands, we determined the capacity of VLA-4, expressed by these different T cells, to bind VCAM-1, a ligand of VLA-4 (24) (Fig. 3). VLA-4 can be activated to bind VCAM-1 by the addition of PMA or by specific anti-β1 antibodies that induce the high affinity state of VLA-4, resulting in enhanced binding of VLA-4 to VCAM-1 and/or FN (10, 12, 13). Despite the fact that VLA-4 was not equally expressed on all T cells (Table 1), all T cells used in this study (LFA-1$^+$/L16$^+$, LFA-1$^+$/L16$^-$, and LFA-1$^-$) could be induced by an anti-β1 antibody (TS2/16; 10) or by PMA (data not shown) to bind to VCAM-1 or FN (data not shown). This induced interaction was completely VLA-4 mediated, since anti-VLA-4α antibodies blocked the interaction. These data indicate that in contrast to LFA-1, the VLA-4 receptor on all T cells used in this study can become active to bind VCAM-1. Moreover, binding of JS136 T cells to TNF-α-stimulated EC also can be enhanced by anti-β1 antibodies (10). Although the increase in the total number of cells bound is limited (from 50 to 60%), this is associated with a clear shift from LFA-1-mediated adhesion to a VLA-4/VCAM-1–mediated adhesion to EC (Fig. 4). However, if both LFA-1 and VLA-4 are activated through the addition of PMA, JS136 T cells primarily use LFA-1 and not VLA-4 to bind EC, indicating that the LFA-1 molecule, when activated, dominates VLA-4 in T cell–EC interaction (Fig. 4). The addition of PMA to CEM

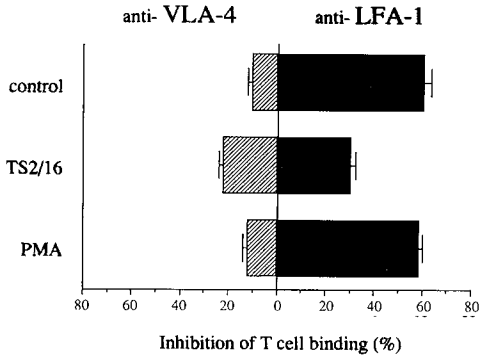

Figure 4. Contribution of LFA-1 and VLA-4 to TS2/16-induced JS136 T cells binding to TNF-α–EC. ^{51}Cr-labeled T cells were allowed to adhere for 30 min at 37°C to 24-h TNF-α-stimulated EC. Adhesion was induced by the addition of anti-β1 antibody TS2/16 (10 μg/ml) or PMA (50 ng/ml). Basal adhesion of 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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