Four Molecular Pathways of T Cell Adhesion to Endothelial Cells: Roles of LFA-1, VCAM-1, and ELAM-1 and Changes in Pathway Hierarchy Under Different Activation Conditions

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Abstract. T cell adhesion to endothelium is critical to lymphocyte recirculation and influx into sites of inflammation. We have systematically analyzed the role of four receptor/ligand interactions that mediate adhesion of peripheral human CD4+ T cells to cultured human umbilical vein endothelial cells (HUVEC): T cell LFA-1 binding to ICAM-1 and an alternative ligand ("ICAM-X"), T cell VLA-4 binding to VCAM-1, and T cell binding to ELAM-1. Contributions of these four pathways depend on the activation state of both the T cell and HUVEC, and the differentiation state of the T cell. ELAM-1 plays a significant role in mediating adhesion of resting CD4+ T cells to activated HUVEC. LFA-1 adhesion dominates with PMA-activated T cells but the strength and the predominant LFA-1 ligand is determined by the activation state of the HUVEC; while ICAM-1 is the dominant ligand on IL-1-induced HUVEC, "ICAM-X" dominates binding to uninduced HUVEC. Adhesion via VLA-4 depends on induction of its ligand VCAM-1 on activated HUVEC; PMA activation of T cells augments VLA-4-mediated adhesion, both in the model of T/HUVEC binding and in a simplified model of T cell adhesion to VCAM-1-transfected L cells. Unlike LFA-1 and VLA-4, ELAM-1-mediated adhesion is not increased by T cell activation. Differential expression of adhesion molecules on CD4+ T cell subsets understood to be naive and memory cells also regulates T/HUVEC adhesion. Naive T cell adhesion to HUVEC is mediated predominantly by LFA-1 with little or no involvement of the VLA-4 and ELAM-1 pathways. In contrast, memory T cells bind better to HUVEC and utilize all four pathways. These studies demonstrate that there are at least four molecular pathways mediating T/HUVEC adhesion and that the dominance/hierarchy of these pathways varies dramatically with the activation state of the interacting cells and the differentiation state of the T cell.

I N the immune system, the process of adhesion is critical to two distinct but related processes to T lymphocyte function: T cell recognition of foreign antigen, and the nonrandom migration or trafficking of lymphocytes to distinct anatomic sites in vivo (27, 41, 49, 51). The attachment of T lymphocytes to endothelial cells and subsequent migration between endothelial cells into the surrounding tissue is critically important to lymphocyte migration. Evidence from a number of studies suggest that at least four molecular pathways of adhesion mediate T cell binding to cultured human umbilical vein endothelial cells (HUVEC)[1] (illustrated

schematically in Fig. 1). The LFA-1 integrin (also CD11a/ CD18) has been shown to mediate lymphocyte binding to HUVEC by binding its well-known ligand ICAM-1 (10, 26, 28) and an undefined ligand, designated in this paper "ICAM-X" (10). An additional LFA-1 ligand, ICAM-2, has been cloned from HUVEC (50), but the functional role of ICAM-2 in T/HUVEC adhesion awaits further analysis. Various studies suggesting that T/HUVEC adhesion can also be mediated by an LFA-1-independent mechanism (1, 10, 39) have been confirmed by the identification of two LFA-1-independent pathways of T/HUVEC adhesion. One pathway is mediated by the inducible endothelial cell adhesion molecule VCAM-1 (6, 33, 36, 39); the lymphocyte molecule that binds VCAM-1 has recently been identified as the VLA-4 (also $\alpha 4\beta 1$) integrin (6, 12, 39), which also mediates adhesion to fibronectin (13, 16, 45, 56). We have recently characterized a novel pathway of T/HUVEC adhesion involving the inducible endothelial cell surface protein ELAM-1 (15,
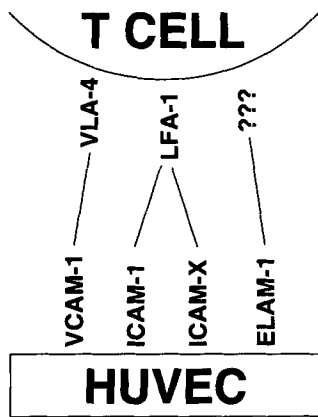

Figure 1. Four molecular pathways of adhesion involved in T cell binding to cultured HUVEC. T cell adhesion to activated HUVEC expressing multiple adhesion molecules is illustrated (see text for details). An undefined ELAM-1 ligand on T cells is identified by *???*.

43), which also mediates granulocyte adhesion to activated HUVEC (3, 32). Thus, at least four adhesion pathways appear to mediate T/HUVEC adhesion: LFA-1/ICAM-1, LFA-1/"ICAM-X," VLA-4/VCAM-1, and ELAM-1.

Several independent lines of evidence suggest that T cell adhesion to endothelial cells can be regulated in multiple ways. First, activation of HUVEC by inflammatory cytokines such as IL-1β and TNF-α results in a rapid increase in expression of ICAM-1 and induced expression of both VCAM-1 and ELAM-1 on the endothelial cell surface (2, 10, 33, 35). The increased expression of these adhesion molecules on activated endothelium is thought to represent a mechanism by which various leukocyte populations can be rapidly recruited into a site of inflammation or tissue injury (20, 32, 51, 53, 57).

Second, the activation state of the T cell is particularly critical in regulating the function of integrins such as LFA-1 and VLA-4 (44, 49). Dustin and Springer first demonstrated that while LFA-1-mediated adhesion of resting T cells to ICAM-1 is minimal, activation of the T cell with the phorbol ester PMA or cross-linking of the T cell receptor results within minutes in strong LFA-1-mediated adhesion without a change in levels of cell surface LFA-1 (11). Similar results have been shown for the VLA-4, VLA-5, and VLA-6 integrins (44, 45), suggesting a common mode of activation-dependent regulation of integrins on human T cells.

Third, an additional mode of regulation of T cell adhesion is related to changes in the level of adhesion molecule expression as a consequence of T cell differentiation. A number of adhesion molecules, including LFA-1 and VLA-4, are differentially expressed on reciprocal subsets of CD4+ T cells defined as "naive" and "memory" cells (37, 38, 44, 45). The greater expression of adhesion molecules on memory T cells is associated with greater memory cell adhesion to purified ligands such as ICAM-1, fibronectin, and laminin, and to other cells (7, 9, 34, 44, 45).

The aim of this study was to integrate these independent findings and systematically analyze how these three modes of regulation interact and affect the adhesion of human peripheral CD4+ T cells to HUVEC. The results show that the ensemble of molecular pathways used in any situation is determined by the combination of these three parameters. Furthermore, we demonstrate an important role for ELAM-1 in adhesion of resting T cells to activated HUVEC and demonstrate for the first time activation-dependent regulation of VLA-4 adhesion to VCAM-1.

Materials and Methods

Cells and Culture Reagents

HUVEC were isolated and cultured as previously described (15) in M199 media containing 20% FCS (Hyclone Laboratories, Logan, UT), 90 μg/ml preservative-free porcine heparin, 20 μg/ml endothelial cell growth supplement, and antibiotics. All HUVEC were used fresh at passage 2 or less. Mouse L cells were cultured by standard procedures in IMEM media (Biofluids, Inc., Rockville, MD) containing 5% FCS and antibiotics. Resting human peripheral CD4+ T cells are isolated from normal donors by rigorous negative immunoselection with magnetic beads as previously described (18). Negative selection is performed with a cocktail of mAbs against HLA class II on B cells, activated T cells, and monocytes (IVA12), CD20 on B cells (1F5), CD16 on NK cells (3G8), CD11b on monocytes (NIH11b-1), CD14 on monocytes (MMA), glycophorin on erythrocytes (10F7), and CD8 (B9.8); purity of the CD4+ T cells was >98%. Presence of the HLA class II mAb IVA12 excluded the normal low percentage of activated T cells from the final isolated cell population. Purified CD4+ CD45RA+ naive and CD4+CD45R0+ memory T cells were isolated by the same procedure with the same mAb cocktail except the CD45R0 mAb UCHL1 was added to negatively select memory T cells and the CD45RA mAb G1-15 was added to negatively select naive T cells.

mAbs

All of the following mAbs were used as dilutions of ascites fluid: CD20 mAb 1F5 and CD45RA mAb G1-15 (24), CD16 mAb 3G8 (D. Segal, National Institutes of Health, Bethesda, MD), CD14 mAb MMA and glycophorin mAb 10F7 (American Type Culture Collection, Rockville, MD), CD8 mAb B9.8 (B. Malissen, Centre d'Immunologie, Marseilles), and the CD45R0 mAb UCHL1 (48). All of the following mAbs were used as purified IgG: HLA class II mAb IVA12 (ATCC), CD11b mAb NIH11b-1 (19), LFA-1 α chain mAb MHM24 (17), LFA-1 β chain mAb MHM23 (17), VCAM-1 mAb 2G7 (15), ELAM-1 mAb 7A9 (15), CD45 mAb NIH45-2 (43), ICAM-1 mAbs W-CAM-1 (4) and 84H10 (26), VLA-4 α chain mAb L25 (8, 52), VLA β chain mAb MAB 13 (30), and the VLA-5 α chain mAb MAB 16 (30).

Flow Microfluorometry

Flow microfluorometry of resting and IL-1-activated HUVEC was performed as previously described (15) using a flow cytometer model 541 (Coulter Electronic, Hialeah, FL) equipped with an argon laser and a quartz flow cell.

Generation of VCAM-1 L Cell Transfectants

Mouse L cell transfectant clone LVE3 was isolated by cotransfection of L cells with separate plasmids containing VCAM-1 and ELAM-1 cDNAs, and pRSVNeo by the CaPO₄ coprecipitation method. Stable transfectant clones were isolated by selection with G418 antibiotic, and tested for membrane expression of VCAM-1 and ELAM-1 by antibody binding with the 2G7 and 7A9 antibodies, respectively (15). One clone, LVE3, showed binding of anti-VCAM-1 (2G7) mAb but not of anti-ELAM-1 (7A9) mAb. This clone also did not produce detectable levels of ELAM-1 mRNA by Northern analysis (not shown).

Cell Adhesion Assays

Binding of CD4+ T cells to HUVEC was assessed as previously described (15, 43). Briefly, HUVEC were plated onto gelatin precoated 24-well plates (Costar Corp., Cambridge, MA) and cultured for 48 hours or until confluent; L cells were plated onto uncoated 24-well plates and cultured to confluence. HUVEC were activated by exposure to 1 ng/ml IL-1β in medium (RPMI/10% FCS) for 4 h at 37°C and then washed once with medium immediately before addition of T cells. CD4+ T cells were labeled with Cr-51 and 300,000 T cells were added to each well in a final volume of 300 μl. For assessing adhesion of acutely activated T cells, CD4+ T cells were preactivated by incubation for 20 min with 10 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) before washing and addition to the HUVEC monolayer. Where blocking by mAbs was assessed, binding was assessed in the continuous presence of antibody; all mAbs were used at a saturating concentration of 10 μg/ml, which has been shown in previous studies to maximally inhibit the relevant adhesive interaction (15, 44–46, 55). Plates were incubated for
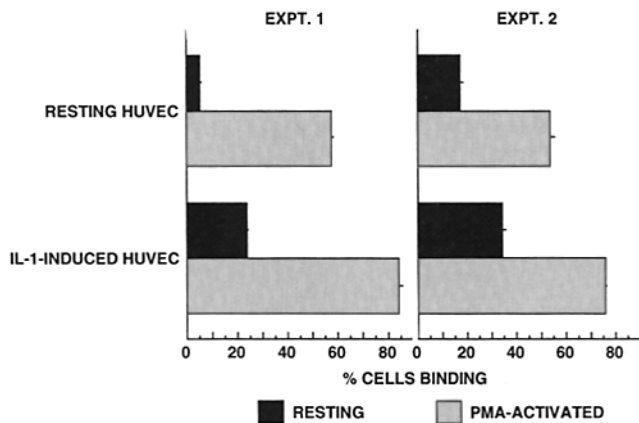
Figure 2. CD4+ T cell adhesion to HUVEC is dependent on the activation state of both cell types. Adhesion of Cr-51-labeled resting (*solid bars*) or PMA-activated (*shaded bars*) CD4+ T cells to resting HUVEC or HUVEC preexposed for 4 h to IL-1β (*IL-1 induced*) was assessed as described in Materials and Methods. Results from two independent experiments using CD4+ T cells from different donors is presented. Data are expressed as mean percent of cells binding from duplicate wells.

30 minutes at 37°C, and then gently washed twice with RPMI/10% FCS media at room temperature to remove nonadherent T cells. Contents of each well containing adherent T cells were lysed with 300 μl of 1% Triton X-100 and γ-emissions were counted. Data are expressed as mean percent of cells binding. No differences in overall adhesion, utilization of adhesion pathways under different activation conditions, or phenotype of adhesion molecules was observed between freshly isolated CD4+ T cells and purified CD4+ T cells that were cryopreserved in liquid nitrogen and thawed before use. All experiments were performed a minimum of three times using different donor T cells and HUVEC; data presented represent results from one or two representative experiments.

Results

T Cell Adhesion to Endothelium Is Dependent on the Activation State of Both Cell Types

Binding of resting peripheral blood CD4+ T cells to early passage cultured HUVEC was assessed using an in vitro adhesion assay (15). Fig. 2 shows that there is minimal adhesion of resting CD4+ T cells to resting HUVEC. However, pretreatment of HUVEC for 4 h with IL-1β results in the induced expression of the adhesion molecules VCAM-1 and ELAM-1 and increased expression of ICAM-1 (Fig. 3). These phenotypic changes in adhesion molecule expression on activated HUVEC are associated with increased binding of resting CD4+ T cells (Fig. 2). While activation of HUVEC results in increased T cell binding, acute activation of the CD4+ T cells results in even greater T/HUVEC adhesion. Preactivation of CD4+ T cells for 20 min with the phorbol ester PMA results in increased adhesion to both resting and IL-1-induced HUVEC compared to resting CD4+ T cells (Fig. 2). Thus, T cell adhesion to HUVEC is dynamically regulated by the activation state of both interacting cell types.

The LFA-1 and VCAM-1 Pathways Mediate Adhesion of PMA-activated T Cells to HUVEC

mAb blocking studies were used to define the contribution of the LFA-1, VCAM-1, and ELAM-1 adhesion pathways to the binding of PMA-activated CD4+ T cells to HUVEC. Adhesion of PMA-activated T cells to resting HUVEC is in-
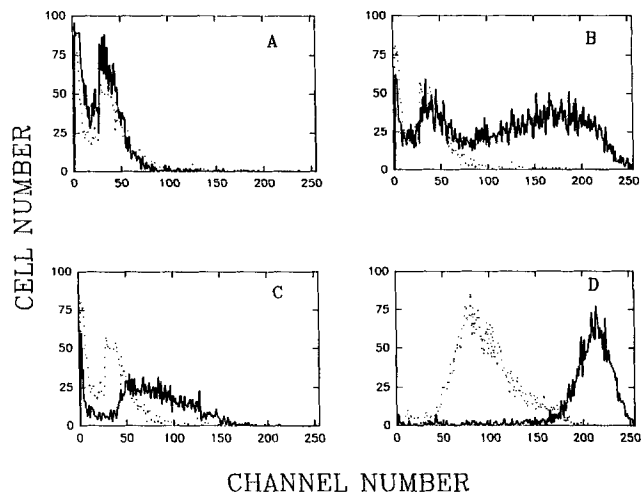

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Figure 3. Activation of HUVEC with IL-1β results in increased ICAM-1 expression and induced expression of VCAM-1 and ELAM-1. Expression of VCAM-1 (*B*), ELAM-1 (*C*), and ICAM-1 (*D*) on resting (*dotted lines*) and IL-1-induced (*solid lines*) HUVEC was assessed by flow cytometry as described in Materials and Methods. Background binding in the presence of the second step goat anti-mouse Ig FITC reagent only is shown in *A*. mAbs used were 2G7 (*VCAM-1-specific*), 7A9 (*ELAM-1-specific*), and 84H10 (*ICAM-1-specific*).

hibited by an LFA-1 mAb alone while neither a VCAM-1-specific or an ELAM-1-specific mAb alone has any inhibitory effect (Fig. 4, *C–E*). The lack of effect of VCAM-1 and ELAM-1 mAbs on binding to resting HUVEC is not surprising, since resting HUVEC do not express either VCAM-1 or ELAM-1 (Fig. 3). Pairwise combinations of LFA-1, VCAM-1, and ELAM-1 mAbs along with all three mAbs together fail to show greater inhibition than seen with the LFA-1 mAb alone (*C* vs. *F–I*, Fig. 4), suggesting that the LFA-1 pathway predominates in mediating adhesion of PMA-activated T cells to resting HUVEC.

Activation of HUVEC with IL-1 results in the induction of VCAM-1 and ELAM-1 expression (Fig. 3). Compared with the inhibition of PMA-activated T cell adhesion to resting HUVEC, the LFA-1 mAb is not as effective in inhibiting adhesion to IL-1-induced HUVEC (Fig. 4 *C*); neither the VCAM-1 or ELAM-1 mAbs alone show any significant inhibition of binding. However, combinations of LFA-1, VCAM-1, and ELAM-1 mAbs clearly demonstrate a contribution of VCAM-1 and, to a lesser extent, ELAM-1. A role for VCAM-1 is shown by the fact that inhibition of binding by the combination of LFA-1 and VCAM-1 mAbs is greater than either mAb alone (compare *F* and *C*, Fig. 4). Furthermore, binding is maximally inhibited by the combination of LFA-1, VCAM-1, and ELAM-1 mAbs, suggesting some involvement of the ELAM-1 pathway (*I*, Fig. 4). Although some adhesion is still detectable in the presence of LFA-1, VCAM-1 and ELAM-1 mAbs, such as in PMA-activated T cell binding to resting HUVEC, we have not been able to further inhibit adhesion with the addition of mAbs specific for other adhesion molecules, such as CD2 and CD44 (data not shown).

Further studies aimed at defining receptor/ligand relationships show that a VLA-4 mAb is as effective as a VCAM-1 mAb in blocking PMA-activated T cell adhesion to IL-1-induced HUVEC (*D* vs. *F* in Fig. 5), suggesting that additional
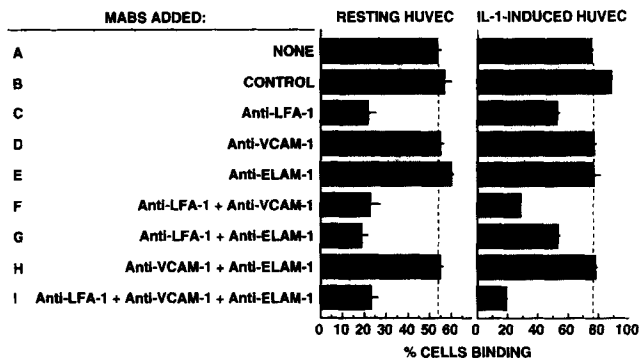
Figure 4. Binding of PMA-activated CD4+ T cells to HUVEC involves multiple adhesion pathways mediated by LFA-1, VCAM-1, and ELAM-1. Binding of PMA-activated CD4+ T cells to resting (*left*) or IL-1-induced HUVEC (*right*) was assessed in the presence of the indicated mAbs. Adhesion was restricted to specific pathways (Fig. 1) by adding mAbs to block the other pathways. Binding was assessed in the continuous presence of the indicated mAbs at a saturating concentration of 10 µg/ml for each mAb, except for the control mAb NIH45-2, which was used at 20 µg/ml. Equivalent blocking was seen with the LFA-1 α chain mAb MHM24 (as shown in the figure) or the LFA-1 β chain mAb MHM23 (not shown). Inhibition seen with pairwise combinations of LFA-1, VCAM-1, and ELAM-1 mAbs was unaffected by the addition of a control mAb, the VLA-5-specific mAb MAB 16 (30). Furthermore, inhibition was not increased by the addition of MAB 16 to the maximally inhibitory combination of LFA-1, VCAM-1, and ELAM-1 mAbs (data not shown). Data are expressed as mean percent of cells binding from duplicate wells.

molecules on the T cell other than VLA-4 are not involved in VCAM-1-mediated adhesion. In contrast, mAbs specific for the LFA-1 ligand ICAM-1 are not as effective as an LFA-1 mAb in blocking binding of PMA-activated T cells to either resting or IL-1-induced HUVEC (*C* vs. *D*, Fig. 5). Even though the two ICAM-1 mAbs used potently inhibit LFA-1/ICAM-1 interactions at the concentrations used (4, 42, 55,
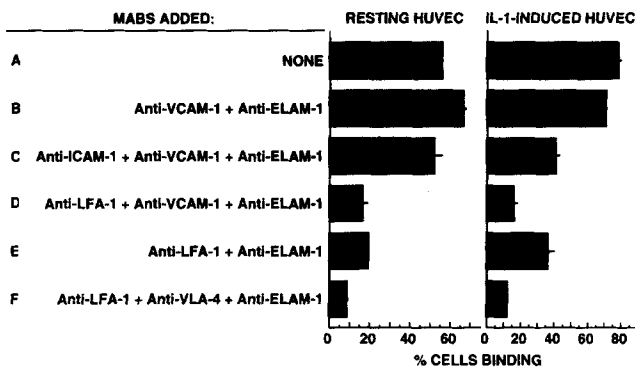

Figure 5. Roles of VLA-4 and ICAM-1 in mediating adhesion of PMA-activated CD4+ T cells to HUVEC. Binding of PMA-activated CD4+ T cells to resting (*left*) and IL-1-induced (*right*) HUVEC was assessed in the presence of the indicated combinations of mAbs to restrict binding to a specific adhesion pathway (Fig. 1). Binding was assessed in the continuous presence of mAb at a saturating concentration of 10 µg/ml for each mAb. Comparable blocking results were obtained with two inhibitory ICAM-1 mAbs, W-CAM-1 (shown) and 84H10 (not shown). Data are expressed as mean percent of cells binding from duplicate wells.
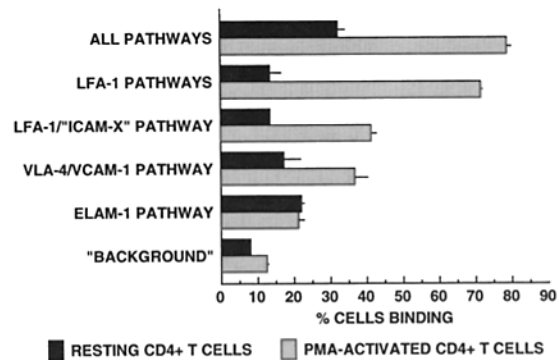

Figure 6. Acute T cell activation increases LFA-1- and VLA-4- but not ELAM-1-dependent adhesion to IL-1-induced HUVEC. Binding of resting (*solid bars*) or PMA-activated (*shaded bars*) CD4+ T cells to IL-1-induced HUVEC was restricted to the indicated adhesion pathways by the inclusion of the following combinations of mAbs: VCAM-1 and ELAM-1 mAbs to isolate the LFA-1 pathway; ICAM-1, VCAM-1, and ELAM-1 mAbs to isolate the LFA-1/"ICAM-X" pathway; LFA-1 and ELAM-1 mAbs to isolate the VLA-4/VCAM-1 pathway; LFA-1 and VCAM-1 mAbs to isolate the ELAM-1 pathway, and a combination of LFA-1, VLA-4, and ELAM-1 mAbs to maximally inhibit binding (*background*). Data are expressed as mean percent of cells binding from duplicate wells.

and data not shown), both were ineffective in blocking binding to resting HUVEC, suggesting that LFA-1-mediated adhesion to resting HUVEC is mediated by LFA-1 binding to another ligand ("ICAM-X"). IL-1-activation of HUVEC, which augments ICAM-1 expression (Fig. 3), results in increased utilization of the LFA-1/ICAM-1 pathway, since an ICAM-1 mAb can now significantly block binding (*B* vs. *C*, Fig. 5).

Acute T Cell Activation Increases LFA-1- and VCAM-1-But Not ELAM-1-dependent Adhesion

To determine which pathways mediate the increased binding of activated T cells to HUVEC, binding of resting and PMA-activated T cells to activated HUVEC was restricted to individual adhesion pathways by adding the appropriate mAb combinations to block each of the other pathways. Assessment of resting T cell adhesion to activated HUVEC shows that while each pathway plays some role, the strongest binding is seen via the ELAM-1 pathway (Fig. 6). Acute activation of T cells with PMA results in increased adhesion via both LFA-1 and VCAM-1, although LFA-1 is clearly the dominant adhesion pathway used by PMA-activated T cells. Furthermore, PMA activation increases T/HUVEC adhesion measured in the presence of an ICAM-1 mAb added to VCAM-1 and ELAM-1 mAbs, suggesting that adhesion via LFA-1 binding to "ICAM-X" is also activation-dependent. In contrast, PMA activation does not result in increased adhesion via ELAM-1. The activation-dependent increase in T cell adhesion to HUVEC via the LFA-1 and VLA-4 integrins occurs without a change in the level of cell surface expression of LFA-1 and VLA-4 (data not shown) (11, 45).

Differential Binding of Naive and Memory CD4+ T Cells to HUVEC

Differential expression of isoforms of the leukocyte surface molecule CD45 defines two reciprocal subsets of CD4+ T

Figure 9. Activation-dependent binding of CD4+ T cells to a VCAM-1 L cell transfectant. Adhesion of resting (solid bars) and PMA-activated (shaded bars) CD4+ T cells to an untransfected L cell control and the VCAM-1+ L cell transfectant LVE3 was assessed as described in Materials and Methods. Data are expressed as the mean percent of cells binding from triplicate wells.
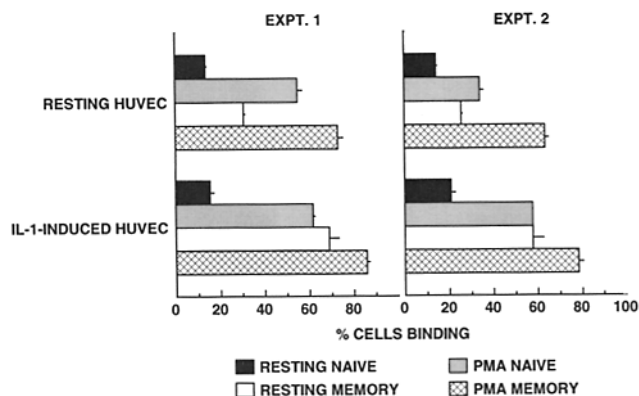
Figure 7. Binding of purified naive and memory CD4+ T cells to resting and IL-1-induced HUVEC. Naive (CD4+CD45RA+) and memory (CD4+CD45RO+) T cells were isolated from a representative donor and assessed for binding to resting and activated HUVEC; purity of subsets was >95% as determined by flow cytometric analysis. Binding of resting naive (solid bars) and memory (open bars) CD4+ T cells and PMA-activated naive (shaded bars) and memory (hatched bars) cells is shown. Results of two independent experiments using naive and memory cells isolated from different donors are presented.

cells (37, 38, 44). The subset expressing the CD45RA isoform consists of resting peripheral T cells that have not been previously activated ("naive" cells). The reciprocal subset expresses the CD45R0 isoform and represent cells that have been previously activated, typically by exposure to antigen, and have reverted back to a resting state ("memory" cells). Analysis of these subsets has shown that in addition to differential expression of CD45 isoforms, there is increased expression of a number of adhesion molecules, including LFA-1 and VLA-4, on memory T cells (44, 45). Fig. 7 shows that the greater expression of adhesion molecules on memory T cells is relevant to T/HUVEC interactions. First, resting purified memory CD4+ T cells bind greater than do resting purified naive CD4+ T cells to either resting or IL-1-induced

HUVEC. Second, acute activation of purified naive and memory T cells with PMA results in increased adhesion of both subsets to HUVEC, although PMA-activated memory T cells still show greater adhesion to HUVEC than do PMA-activated naive T cells.

Adhesion Pathways Utilized by Naive and Memory T Cells in Binding to HUVEC

mAb blocking studies were performed to determine if the LFA-1, VCAM-1, and ELAM-1 pathways were equivalently used by PMA-activated naive and memory T cells in binding to HUVEC. Adhesion of both PMA-activated naive and memory T cells to resting HUVEC is blocked by an LFA-1 mAb but not VCAM-1 or ELAM-1 mAbs (compare C, D, and E in Fig. 8). Binding of both subsets via LFA-1 is only partially
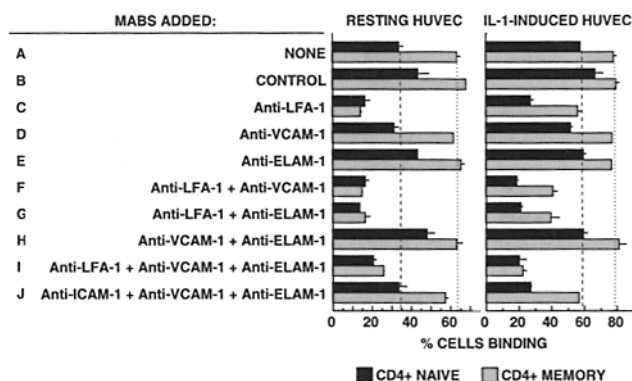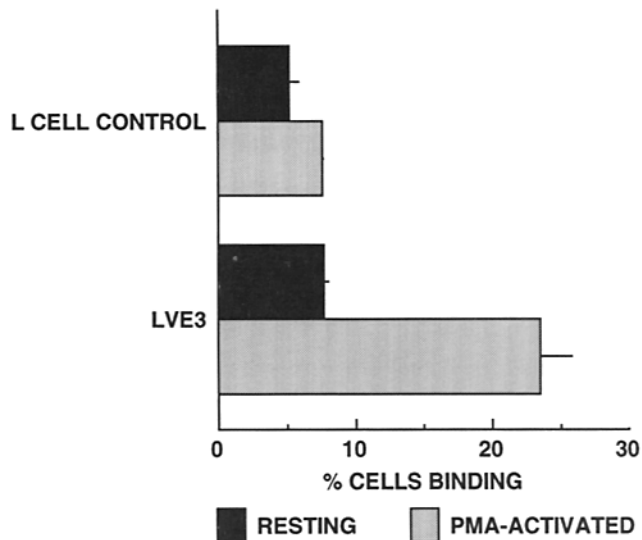

Figure 8. Differential utilization of the LFA-1, VCAM-1, and ELAM-1 pathways by naive and memory CD4+ T cells in adhesion to HUVEC. Binding to resting and IL-1-induced HUVEC of PMA-activated naive (solid bars) and memory (shaded bars) CD4+ T cells isolated from a representative donor in the presence of the indicated mAbs was assessed as described in Materials and Methods. MAbs were used at the concentrations indicated in Fig. 4 and 5. The LFA-1 mAb was the LFA-1 α chain-specific mAb MHM24 and the ICAM-1 mAb used was W-CAM-1.

Table I. Specificity of CD4+ T Cell Adhesion to VCAM-1 L Cell Transfectant LVE3

mAb added	Specificity	% Inhibition of binding
None	–	0
2G7	VCAM-1	75
MAB 13	VLA β chain	100
L25	VLA-4 α chain	100
MAB 16	VLA-5 α chain	10
MHM24	LFA-1	13
W-CAM-1	ICAM-1	1
7A9	ELAM-1	19

Binding of PMA-activated CD4+ T cells to the VCAM-1+ L cell transfectant LVE3 was performed as described in Materials and Methods. Adhesion was measured in the continuous presence of the indicated mAbs used at a saturating concentration of 15 μg/ml. Background binding of PMA-activated CD4+ T cell adhesion to an untransfected L cell control was subtracted from the specific binding; the indicated percent inhibition of binding in the presence of the indicated mAb was calculated relative to binding in the absence of any mAb (top line).
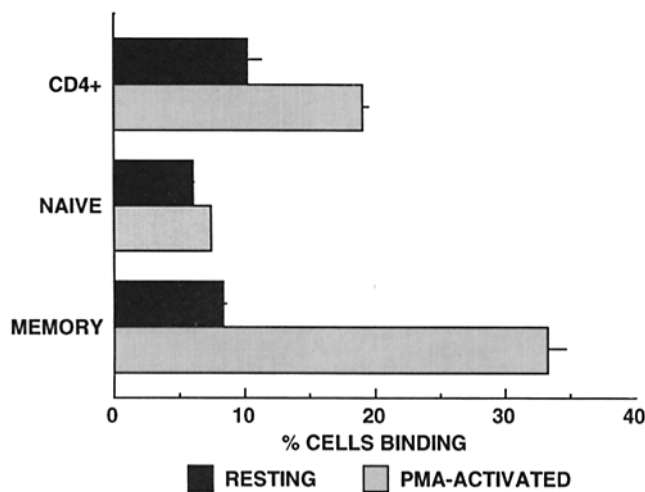
Figure 10. Differential binding of naive and memory CD4+ T cells to a VCAM-1 transfectant. Binding to the VCAM-1 L cell transfectant LVE3 of three populations of T cells isolated from the same donor was assessed: CD4+, CD4+ naive *(CD4+CD45RA+)*, and CD4+ memory *(CD4+CD45RO+)*. Adhesion of resting *(solid bars)* and PMA-activated *(shaded bars)* T cells is shown. Binding of all CD4+ cells to an untransfected L cell control was <10% and has not been subtracted from the values shown. Data are expressed as the mean percent of cells binding from triplicate wells.

blocked by an ICAM-1 mAb, suggesting that PMA-activated naive and memory T cells both use an additional LFA-1 ligand other than ICAM-1 to bind to resting HUVEC (*H, I,* and *J* in Fig. 8). Binding of PMA-activated naive T cells to activated HUVEC is also substantially inhibited by an LFA-1 mAb; there is little evidence for a contribution by VCAM-1 and ELAM-1 in naive T cell binding to activated HUVEC since neither VCAM-1 or ELAM-1 mAbs alone inhibit adhesion (*D* and *E* in Fig. 8) and the combination of either with an LFA-1 mAb fails to show significantly greater inhibition than the LFA-1 mAb alone (*F* and *G* in Fig. 8). In addition, an ICAM-1 mAb is almost as effective as an LFA-1 mAb in blocking PMA-activated naive T cell adhesion to activated HUVEC, suggesting that LFA-1/ICAM-1 is the predominant pathway used by PMA-activated naive T cells to interact with activated HUVEC (*I* vs. *J* in Fig. 8).

In contrast, these studies suggest the utilization of multiple additional adhesion pathways by PMA-activated memory T cells. While an LFA-1 mAb alone partially inhibits memory cell adhesion to IL-1-induced HUVEC, the addition of either a VCAM-1 or an ELAM-1 mAb results in greater inhibition of binding (*F* and *G* vs. *C* in Fig. 8). In addition, the combination of an LFA-1, VCAM-1, and an ELAM-1 mAb maximally inhibits adhesion (*I* in Fig. 8), indicating that all three molecules are involved in binding of PMA-activated memory T cells to activated HUVEC. Furthermore, in contrast to the results obtained with naive T cells, LFA-1-mediated binding of PMA-activated memory T cells is only partially inhibited by an ICAM-1 mAb (*I* vs. *J* in Fig. 8), suggesting that activated memory T cells bind to both ICAM-1 and "ICAM-X" on activated HUVEC.

Activation-dependent Binding of CD4+ T Cells to a VCAM-1 L Cell Transfectant

Our analysis of T/HUVEC adhesion suggests that VLA-4-

mediated binding to VCAM-1, like VLA-4 binding to its extracellular matrix ligand fibronectin, is increased by acute activation of the T cell (Fig. 6). To confirm this finding in a simpler system not involving multiple inhibitory mAbs, we have analyzed the adhesion of resting and PMA-activated CD4+ T cells to mouse L cells transfected with a VCAM-1 cDNA clone. Fig. 9 shows that both resting and PMA-activated CD4+ T cells bind minimally to an untransfected L cell control. While resting T cells also bind minimally to the VCAM-1 transfectant, adhesion of PMA-activated T cells can be clearly observed (Fig. 9). This binding is specific, since it can be blocked by mAbs specific for VCAM-1, the VLA-4 α chain, and the common VLA β chain but not by other relevant mAbs specific for LFA-1, VLA-5, ELAM-1, and ICAM-1 (Table I).

Generation of functional VCAM-1 transfectants also enabled us to assess the utilization by purified naive and memory CD4+ T cells of the VLA-4/VCAM-1 pathway in isolation. The results shown in Fig. 10 confirm the results obtained by antibody blocking in our T/HUVEC adhesion assay (Fig. 8). While memory T cell adhesion to the VCAM-1 transfectant is dramatically increased by PMA-activation, PMA-activation of naive CD4+ T cells results in a minimal change in binding. These results provide further evidence suggesting that memory cells utilize the VLA-4/VCAM-1 adhesion pathway after acute activation with PMA to a much greater extent than naive CD4+ T cells.

Discussion

The interaction of T lymphocytes with endothelial cells is critical not only to the normal migration of lymphocytes but also to the mobilization and influx of lymphocytes into sites of inflammation and tissue injury. The results presented here confirm and extend previous independent studies demonstrating that T cell interactions with cultured HUVEC are mediated by multiple adhesion pathways (10, 15, 36, 39, 43). Furthermore, our results demonstrate changes in the dominance or hierarchy of the various multiple pathways mediating T/HUVEC adhesion that depend on the activation state of the T cell and the HUVEC, and the differentiation state of the T cell. The following discussion will focus on: (*a*) the multitude of adhesion pathways that mediate T/HUVEC interaction; (*b*) the modes of regulation that play a role in modulating T/HUVEC interaction; (*c*) the significance of the differential binding of T cell subsets to HUVEC; and (*d*) the relevance of these findings to lymphocyte migration and function in vivo.

The hierarchy of the four adhesion pathways that mediate T/HUVEC adhesion under the different activation conditions analyzed in this study are summarized in Table II. We highlight the following conclusions from our data. First, we have shown that the adhesion of resting CD4+ T cells to IL-1-induced HUVEC is mediated by independent pathways of adhesion involving LFA-1, VCAM-1, and a newly described pathway of T cell adhesion mediated by ELAM-1 (15, 43, and Fig. 6). Multiple pathways working together may be particularly important for resting T cells, since this would provide the best opportunity for a broad spectrum of T cells to be recruited into an inflammatory site. While a role for all four pathways can be demonstrated for resting T cells, it is interesting to note that the ELAM-1 pathway is as effective as

Table II. Summary of Relative Dominance of Adhesion Pathways Used in T Cell Adhesion to Endothelial Cells

T cell	HUVEC	Overall adhesion	Adhesion via individual pathways				Figures
			LFA-1/ICAM-1	LFA-1/"ICAM-X"	VLA-4/VCAM-1	?/ELAM-1	
CD4+							
Resting	Resting	±					2
Activated	Resting	+ +	±	+ +	−	−	2, 4, 5, and 6
Resting	Activated	+	±	±	±	+	2 and 6
Activated	Activated	+ + +	+ + +	+ + +	+ +	+	2, 4, 5, and 6
CD4+ naive							
Resting	Resting	±					7
Activated	Resting	+ +	±	+	−	−	7 and 8
Resting	Activated	±					7
Activated	Activated	+ +	+ +	±	−	−	7 and 8
CD4+ memory							
Resting	Resting	+					7
Activated	Resting	+ +	±	+ +	−	−	7 and 8
Resting	Activated	+ +					7 and 8
Activated	Activated	+ + +	+ + +	+ + +	+ +	+	7 and 8

Overall adhesion of the indicated combinations of T cells and HUVEC tested in Figs. 2 and 7 is indicated under the column on a relative scale ranging from low (±) to high (+ + +) levels of adhesion. Contribution to adhesion via individual pathways under the different activation conditions studied is indicated on a relative scale ranging from no contribution (±) to very strong contribution (+ + +).

the other three pathways in mediating adhesion (Fig. 6). Thus, this recently described pathway of T/HUVEC adhesion via ELAM-1 may play a particularly critical role in the initial attachment of resting T cells to activated endothelium.

Second, acute activation of the T cells with PMA results in dramatically increased T/HUVEC adhesion (Fig. 2). In contrast to resting T cells, where multiple weak pathways cooperate to mediate binding, PMA-activated T cell adhesion to HUVEC is mediated predominantly via the LFA-1 integrin (Table II). Our results suggest that LFA-1 mediates T/HUVEC adhesion by binding not only to its well characterized ligand, ICAM-1, but also by binding to another ligand, "ICAM-X." Even though the ICAM-1 mAbs used in our studies potently inhibit LFA-1-mediated adhesion to ICAM-1 (4, 42, 55), these mAbs are not as effective as LFA-1 mAbs in inhibiting T/HUVEC adhesion (Fig. 5); our results are consistent with earlier studies of T cell adhesion to HUVEC suggesting the involvement of an additional LFA-1 ligand (10). The LFA-1/"ICAM-X" pathway is particularly dominant in activated T cell binding to resting HUVEC (Fig. 5). This additional LFA-1 ligand is likely to be ICAM-2, which has been reported to be constitutively expressed on resting HUVEC and, in contrast to ICAM-1, is not increased in expression after IL-1 activation (50). Activation of HUVEC results in increased ICAM-1 expression (Fig. 3) and increased LFA-1-dependent adhesion via its more well-characterized ligand, ICAM-1 (Fig. 5). The functional significance of a switch in LFA-1-mediated T/HUVEC adhesion from ICAM-1 to "ICAM-X" or vice versa remains to be defined.

Third, VLA-4 also plays a role in mediating adhesion of T cells to activated HUVEC by binding its ligand VCAM-1. The existence of LFA-1-independent pathways was first suggested by the inability of LFA-1 mAbs to completely inhibit T/HUVEC binding (10). Furthermore, despite the lack of LFA-1 expression on cells of patients with leukocyte adhesion deficiency (LAD) (1), LAD lymphocytes appear to migrate normally in vivo and are still able to bind to HUVEC in vitro (1, 39). Recent studies have clearly shown that the

VLA-4/VCAM-1 pathway can mediate the adhesion of normal and LAD lymphocytes to HUVEC (6, 12, 36). Although a role for the VLA-4/VCAM-1 pathway can be demonstrated in T/HUVEC adhesion, particularly when both the T cell and HUVEC are activated (Fig. 4), it clearly does not contribute as strongly to the overall adhesion as does the LFA-1 pathway.

The inability of the combination of LFA-1, VCAM-1, and ELAM-1 mAbs to completely inhibit T cell adhesion to HUVEC under certain conditions (Fig. 4) suggests the potential involvement of other receptor/ligand interactions. However, we did not observe any inhibition by mAbs (either alone or together with the maximally inhibitory combination of LFA-1, VCAM-1 and ELAM-1 mAbs) specific for three adhesion molecules that might be expected to play a role in T/HUVEC adhesion (data not shown): (a) LECCAM-1 (also MEL-14 in the mouse, Leu-8 in the human) (5), which has been shown in the mouse to mediate the adhesion of lymphocytes to peripheral lymph node high endothelial venules (14, 47); (b) CD44, which has been implicated in the binding of activated T cells to IL-1-activated HUVEC (31); and (c) CD2, which mediates T cell adhesion to many other cell types by binding its ligand, LFA-3 (27, 41). Thus, while we do not exclude the potential involvement of other receptor/ligand interactions, our results suggest that LFA-1, VCAM-1 and ELAM-1 are the major mediators of T cell adhesion to HUVEC.

Although activation of either the T cell or the endothelial cell results in increased T/HUVEC adhesion, the mechanisms by which activation increases adhesion are clearly distinct. On the endothelial cell, activation by inflammatory cytokines results in the increased expression of ICAM-1 and induction of expression of VCAM-1 and ELAM-1 (Fig. 3). Thus, endothelial cells regulate lymphocyte adhesion by changes in the level of expression of adhesion molecules as a result of cytokine activation. While acute activation of the T cell with PMA also results in increased adhesion via LFA-1 and VLA-4/VCAM-1 (Fig. 6 and Table II), it is due not to increased

expression of LFA-1 and VLA-4 but to a qualitative change in the functional activity of these two integrins. Several groups have shown that integrin-mediated adhesion of T cells to purified ligands such as ICAM-1 and fibronectin and to other cells is dependent on the activation state of the T cell (11, 44, 45, 54). While integrin molecules on resting T cells are minimally active in binding, integrin-mediated adhesion can be dramatically upregulated within minutes by acute activation of the T cell without a change in the quantitative level of integrin cell surface expression. The exact mechanism by which activation regulates integrin function currently remains undefined. Our results show that T cell adhesion to HUVEC via the LFA-1 and VLA-4 integrins, like adhesion to purified ligands, is similarly regulated by the activation state of the T cell. Furthermore, our data suggest that LFA-1-mediated adhesion to its alternative ligand "ICAM-X" is also regulated by T cell activation (Fig. 5).

VLA-4 has both an extracellular matrix ligand, fibronectin (13, 16, 45, 56), and a cell surface ligand, VCAM-1 (12). Our results demonstrate for the first time that VLA-4-mediated adhesion to VCAM-1 is upregulated by acute activation of the T cell (Figs. 6 and 9). Thus, although the binding sites for VCAM-1 and fibronectin on VLA-4 do not appear to be identical (12), a common mode of regulation exists for VLA-4-mediated adhesion to both ligands.

In contrast to the LFA-1 and VLA-4/VCAM-1 pathways, the ELAM-1 pathway is neither increased or decreased by acute T cell activation. This has been demonstrated by showing that both resting and PMA-activated CD4+ T cells bind comparably to IL-1-induced HUVEC when the ELAM-1 pathway is isolated by adding mAbs that block the other pathway (Fig. 6). Furthermore, we have recently shown using purified ELAM-1 that CD4+ T cells, whether resting or activated, specifically adhere to ELAM-1 and this binding is not augmented by PMA activation (43).

The differential expression of an ensemble of adhesion molecules, including LFA-1 and VLA-4, on naive and memory CD4+ T cells is associated with differential adhesion of these T cell subsets to purified ligands (44, 45) and to other cells (7, 9, 34). While both naive and memory T cells express LFA-1 and VLA-4, there is about a twofold greater expression of LFA-1 and a three- to fourfold greater expression of VLA-4 on memory CD4+ T cells (44). Our results show that for any particular activation state of the T cell or the HUVEC, memory T cells bind more strongly to HUVEC than comparable naive T cells isolated from the same donor (Fig. 7).

The greater adhesion of memory T cells to HUVEC is due to increased utilization of all four pathways mediating T/HUVEC adhesion; adhesion of PMA-activated naive T cells is mediated predominantly via the LFA-1 pathways. Our studies with both HUVEC and VCAM-1 transfectants do not show significant VLA-4-mediated adhesion of PMA-activated naive T cells to VCAM-1 (Figs. 8 and 10). This result is surprising, since naive T cells express significant levels of VLA-4 (45). Clearly overall integrin function is not impaired on naive T cells, since significant binding of PMA-activated naive T cells to activated HUVEC occurs via the related integrin LFA-1. This suggests that on naive T cells, VLA-4, in contrast to LFA-1, functions quite poorly in mediating adhesion to HUVEC. Consistent with our studies using purified ELAM-1 (43), we also find no evidence for naive T cell adhesion to HUVEC via ELAM-1 (Fig. 8). The data

in Fig. 7 also show a dramatic increase in adhesion of resting memory T cells after HUVEC activation. Since LFA-1 and VLA-4 function on resting T cells is minimal (Table II), this suggests that the ELAM-1 pathway may be particularly critical in mediating adhesion of resting memory T cells to activated HUVEC (43).

The delineation of the various adhesion molecules involved in T/HUVEC interaction and the ways in which their function is regulated is important for understanding how T cell interactions with endothelium in vivo regulate normal lymphocyte trafficking and influx into sites of inflammation (32, 51). It has been previously shown that memory T cells are preferentially found in tissue and in sites of inflammation (21–23, 34). The increased utilization of multiple adhesion pathways by memory T cells in T/HUVEC binding suggests that compared to naive T cells, memory T cells have multiple mechanisms by which to attach and migrate into tissue. Our results may also be relevant to an understanding of normal lymphocyte trafficking, since studies by Mackay and coworkers have demonstrated differential trafficking patterns of naive and memory T cells in vivo (25). The utilization of multiple adhesion pathways by memory but not naive cells in HUVEC binding may be critical to such differential recirculation of these subsets in vivo. The dramatic effects of T cell activation on the strength of T/HUVEC adhesion further suggests that the small percentage of acutely activated T cells found in the peripheral blood may be particularly likely to migrate into tissue and inflammatory sites.

Why are so many adhesion pathways involved in mediating T/HUVEC adhesion? The overall complexity likely reflects the critical importance of this adhesive interaction to overall lymphocyte function. A complex adhesion system has several important advantages. First, the existence of multiple adhesion pathways provides an important safeguard of redundancy within the system. Such redundancy in adhesion is seen in other critically important cell–cell interactions, such as interactions of cytotoxic T lymphocytes with target cells (40). Second, multiple adhesion pathways allow for increased adhesion through cooperativity. This is particularly evident in adhesion of PMA-activated memory T cells to IL-1-induced HUVEC, where four adhesion pathways (LFA-1/ICAM-1, LFA-1/"ICAM-X," VLA-4/VCAM-1, and ELAM-1) contribute to mediate binding. Nevertheless, studies with transfectants and purified ligand show that VCAM-1 and ELAM-1 are sufficient on their own to mediate adhesion (Fig. 9) (43). Third, different adhesion pathways can be differentially regulated. For example, while the LFA-1 and VCAM-1 pathways are rapidly upregulated after T cell activation, the ELAM-1 pathway is unaffected by the activation state of the T cell. Furthermore, memory but not naive T cells preferentially utilize both the ELAM-1 and VLA-4 adhesion pathways. The nature of the activation signal delivered to HUVEC may also play a role in differential utilization of these pathways, since in vitro studies show that some cytokines, such as IL-4, specifically induce VCAM-1 but not ELAM-1 expression (29). Thus, multiple mechanisms may serve to independently regulate these various adhesion pathways at specific inflammatory sites in vivo. Finally, the existence of multiple adhesion pathways in T/HUVEC interaction may reflect the need for certain molecules to carry out distinct functions that are not mediated by other molecules: some of the pathways may also transduce intercellular signals

to the cell that modulate relevant functional responses, while others may be critical to transendothelial migration.

In summary, we have shown that the hierarchy of the four pathways (LFA-1/ICAM-1, LFA-1/"ICAM-X," VLA-4/VCAM-1, and ELAM-1) mediating T/HUVEC adhesion varies with the activation state of the interacting cells and the differentiation state of the T cell. These results are critical not only to our understanding of lymphocyte migration but also provide important insights into how functionally important cell–cell interactions are regulated by multiple adhesion molecules.

Note Added in Proof. A more detailed description of the LVE-3 cell line can be obtained from Polle, T., W. Newman, G. Raghunathan, and T. Venkat Gopal. 1991. Structural and functional studies of full-length vascular cell adhesion molecule-1(VCAM-1). Internal duplication and homology to several adhesion proteins. *DNA Cell Biol.* In press.

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