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High Affinity Very Late Antigen-4 Subsets Expressed on T Cells Are Mandatory for Spontaneous Adhesion Strengthening But Not for Rolling on VCAM-1 in Shear Flow¹

Chun Chen,* James L. Mobley,²† Oren Dwir,* Frida Shimron,* Valentin Grabovsky,* Roy R. Lobb,‡ Yoji Shimizu,† and Ronen Alon³*  

The very late Ag-4 (VLA-4) integrin supports both rolling and firm adhesion of leukocytes on VCAM-1 under shear flow. The molecular basis for the unique ability of a single adhesion molecule to mediate these versatile adhesive processes was investigated. VLA-4 occurs in multiple activation states, with different affinities to ligand. In this study we tested how these states regulate VLA-4 adhesiveness under shear flow in Jurkat T cells and PBL. VLA-4 on nonstimulated Jurkat cells supported rolling and spontaneous arrest on VCAM-1, whereas a Jurkat activation mutant with reduced VLA-4 affinity failed to spontaneously arrest after tethering to or during rolling on VCAM-1. The contribution of VLA-4 affinity for ligand to rolling and spontaneous arrests on immobilized VCAM-1 was dissected using soluble VLA-4 ligands, which selectively block high affinity states. VLA-4 saturation with ligand completely blocked spontaneous adhesion strengthening post-tethering to VCAM-1, but did not impair rolling on the endothelial ligand. High affinity VLA-4 was found to comprise a small subset of VLA-4 on resting Jurkat cells and PBL. This subset is essential for firm adhesion but not for tethering or rolling adhesions on VCAM-1. Interestingly, low and high affinity VLA-4 states were found to mediate similar initial tethering to ligand. High affinity VLA-4, constitutively expressed on circulating T cells, may control their early adhesion strengthening on VCAM-1-expressing endothelium before exposure to vascular chemokines and activation of additional integrins. The Journal of Immunology, 1999, 162: 1084–1095.

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*Abbreviations used in this paper: VLA-4, very late Ag-4; wt, wild type; FN, fibronectin; LDV, leucine-aspartate-valine; HSA, human serum albumin; LIBS, ligand-induced binding site; 2-DG, 2-deoxyglucose; DVL, aspartate-valine-leucine, sVCAM, soluble VCAM.
on the cell type and is tightly regulated by cell stimulation and differentiation (3). The adhesiveness of VLA-4 is also regulated by its association with the cytoskeleton (17, 21). Elucidation of the mechanisms that contribute to VLA-4-mediated rolling and spontaneous arrest of leukocytes on VCAM-1 will further our understanding of the functional regulation of this key vascular integrin in different inflammatory contexts.

In this study the contribution of the intrinsic affinity of VLA-4 for soluble ligand to its involvement in initiation and propagation of lymphocyte adhesion to VCAM-1 under physiological shear flow was elucidated. A homogeneous cellular system, the lymphoblastoid T cell line Jurkat, was used along with freshly isolated PBL, to address this question. Jurkat cells express a high uniform level of VLA-4 and their integrin regulation and function resemble those in effector VLA-4(high) PBL subsets (22), which may comprise the major migratory populations at chronic sites of inflammation that express VCAM-1 (2). The Jurkat line is one of the most widely investigated T cell models for regulation of integrin function and T cell signaling (23–26). The adhesive properties of VLA-4 were compared in wild-type (wt) Jurkat cells and in a Jurkat mutant with major defects in activation-dependent integrin adhesiveness (22). The mutant does not respond to activating signals downstream of PKC and expresses an altered form of the mitogen-activated protein kinase ERK1 (22). We show here that the mutant also lacks a subset of high affinity VLA-4 that can be found on wt cells. The absence of high affinity VLA-4 on mutant cells was associated with a markedly suppressed adhesion strengthening of the mutant cells on different VLA-4 ligands, but did not abrogate their VLA-4-mediated treading and rolling adhesions on VCAM-1 in shear flow. High affinity VLA-4 subsets that are constitutively expressed on circulating lymphocytes may provide a regulatory mechanism for their arrest on inflamed VCAM-1-expressing endothelial sites independent of activation signals displayed on these sites.

Materials and Methods

Abs and reagents

The purified Ig fractions of the following mAbs were used: 4B9, which functionally blocks VCAM-1; HP 1/2, which blocks α5 subunit binding to VCAM-1 and fibronectin (FN) (27); BSG10, which binds the α4 integrin subunit but does not block its binding to ligand (28); TS2/16, which binds and cytochalasin B were purchased from Calbiochem (La Jolla, CA). Sigma (St. Louis, MO). Human serum albumin (HSA; fraction V), PMA, and the mutants also lacks a subset of high affinity VLA-4 that can be functionally blocks VCAM-1; HP 1/2, which blocks a subset of high affinity VLA-4 that can be described previously (22). The wild-type and mutant Jurkat cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma), 2 mM l-glutamine, and penicillin/streptomycin (Bio Lab, Jerusalem, Israel). CHO cells transfected with full-length human VCAM-1 were maintained in α-MEM supplemented with 10% dialyzed FCS, 4 mM l-glutamine, and 200 mM n-methotrexate (Sigma). Human PBL (obtained from healthy donors) were isolated from citrate-anticoagulated whole blood by dextran sedimentation and density separation over Ficoll-Hypaque (34). The mononuclear cells thus obtained were washed and further purified on nylon wool. The resulting purified PBL were >90% CD3(T) lymphocytes.

Immunofluorescence flow cytometry

For indirect immunofluorescence, washed cells were resuspended in PBS supplemented with 5% FCS and 5 mM EDTA (PBS-EDTA) and incubated at 4°C for 60 min with A4-PU34 ascites fluid (diluted 1:200), HPI/2 mAb (at 10 μg/ml), or preimmune mouse IgG (a control for background staining; Ceder, South San Francisco, CA). The cells were then washed and incubated for an additional 30 min at 4°C with FITC-conjugated goat anti-mouse Ig (Zymed). Washed cells were resuspended in PBS supplemented with 0.05% sodium azide and immediately analyzed in a FACSscan flow cytometer (Becton Dickinson, Erembodegem, Belgium).

The induction of ligand-induced binding site (LIBS) epitopes by soluble ligands tested by incubation (5 min, 24°C) of PBL-EDTA-washed cells (5 × 10^7 cells/ml) in binding medium (HBSS containing 2 mg/ml BSA and 10 mM HEPES, pH 7.4, supplemented with Ca^{2+} and Mg^{2+}, each at 1 mM) in the presence of LDL- or DVL-containing octapeptides. High affinity recognition of LDV coincides with high VLA-4 affinity for VCAM-1: both ligands bind the integrin at nearly identical sites, and their binding properties are similarly modulated by integrin activation. The LIBS-specific anti-β1 mAb, 9EG7, was added at 10 μg/ml for a short incubation period (5 min, 24°C), followed by a second incubation of 60 min at 4°C. Unbound primary mAb was removed by washing the cells with PBS supplemented with 5% FCS, and the cells were stained with FITC-labeled goat anti-mouse Ig as described above. Induction of the 9EG7 epitope (determined in mean fluorescense units and corrected for background 9EG7 staining that was detected in cells suspended in binding medium in the absence of soluble VLA-4 ligands) was expressed as a percentage of the mean fluorescence intensity of α-specific staining with mAb HP1/2.

Immunoelectron microscopy

Localization of VLA-4 was assessed by immunoelectron microscopy, as previously described (35). Briefly, cultured Jurkat cells were washed with PBS containing 5 mM EDTA and resuspended in PBS supplemented with 1 mM CaCl_2 and 1 mM MgCl_2. The following fixation and immunolabeling steps were conducted at 22°C. The cell suspension was diluted in 0.1 M phosphate buffer, pH 7.4, containing 2% p-formaldehyde and 0.05% glutaraldehyde. After 20 min, the cells were washed (three times) with HBSS containing 1 mM MgCl_2, 1 mM CaCl_2, and 10 mM HEPES, pH 7.4. They were then incubated (30 min) with the anti-VLA-4 mAb BSG10 (10 μg/ml) in HBSS containing 1% BSA (HBSS/BSA). After washing (three times), cells were incubated (30 min) with rabbit anti-mouse Ig (10 μg/ml) in HBSS/BSA. The cells were washed (three times) and incubated (45 min) with 5-nm gold particle-conjugated protein A (Zymed). After extensive washing with HBSS/BSA, the cells were subjected to an additional fixation (30 min) in 0.1 M sodium cacodylate buffer containing 1.5% glutaraldehyde and 1% sucrose. The cells were then washed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, and stained with aqueous uranyl acetate. Next, the cells were embedded in Epon, sectioned, and stained with aqueous uranyl acetate and lead nitrate. The sectioned cells (40–60 cells/experiment) were examined with a Phillips 410 electron microscope (Phillis, Eindhoven, The Netherlands); only representative cells were photographed.

Laminar flow assays

Analysis of cell tethering. A polystyrene plate on which purified ligand had been adsorbed was assembled in a parallel plate laminar flow chamber (260-μm gap) and mounted on the stage of an inverted phase contrast microscope (Diaphot 300, Nikon, Tokyo, Japan) as previously described (7, 36). The cells to be analyzed were washed twice with cation-free H/H medium (HBSS containing BSA (2 mg/ml) and 10 mM HEPES, pH 7.4) containing 5 mM EDTA, resuspended (10^7 cells/ml, 4°C) in H/H medium,
and kept at 4°C until used. Cells were diluted with room temperature binding medium to a concentration of 10^6 cells/ml and perfused through the flow chamber at the desired shear stress. This stress was generated with an automated syringe pump (Harvard Apparatus, Natick, MA) attached to the outlet side of the flow chamber. Cellular interactions on two different fields of view (each one 0.17 mm^2 in area) were visualized with ×10 objective. Cell images were videotaped with a long integration LIS-700 CCD video camera (Applitec, Holon, Israel) and a Sony SLV E400 video recorder (Sony, Tokyo, Japan). Cell images were manually quantitated by analysis of images directly from the monitor screen.

All adhesive interactions between the flowing cells and the ligand-coated or uncoated (control) substrates were tracked and quantified. Tethering events were defined as adhesive interactions between flowing cells that moved closest to the lower wall of the flow chamber coated with the substrate. Since the majority of tethered cells that spontaneously arrested on VCAM-1 did so within 10 s after its initial adhesive attachment (tethering), the motion of each tethered cell was monitored for 10 s following the initial tether, and four categories of cell tethers were defined. Tethers were defined as transient if cells attached briefly (≤20 s) to the substrate but did not continue to roll on it. Tethers were defined as rolling tethers if they were followed by rolling motions ≥5 s with a velocity of at least 1 μm/s but not more than 10% of the hydrodynamic velocity of a cell freely flowing at the given shear stress. Spontaneous arrests (also referred to as rolling-associated arrests) were defined as cells rolling for 1–10 s post-tethering before coming to a full arrest on the substrate, whereas a fully arrested cell was defined as a cell that remained adherent and stationary for at least 20 s. The last category of tethers, termed immediate arrests, consisted of cells that upon tethering arrested within <0.5 s on the ligand-coated substrate and remained stationary for ≥20 s. Successful transient tethering events separated by ≤200 μm of cell motion at the hydrodynamic velocity were counted as a single tethering event. The number of tethers for each category that occurred within the field of view during a 60-s period of continuous flow was divided by the flux of freely flowing cells that moved through the same field without exhibiting any adhesive interactions. This flux was assessed by running parallel experiments on the same substrates in the presence of EDTA, which inhibited all integrin-mediated interactions with the substrate. For calculations of cell flux, only the fraction of perfused cells that came into close proximity with the substrate and therefore were potentially capable of interacting with the substrate, were considered.

Analysis of cell resistance to detachment and rolling at elevated shear stress. The resistance of an adherent cell to detachment by shear force is a function of the adhesive forces generated by the integrin:ligand bonds at the cell-substrate contact zone (15). Detachment assays were performed with cells that had tethered and accumulated at low shear flow (0.5 dyn/cm^2) for 30 s on VCAM-1-coated plates or that had bound at stasis to ligand-coated plates for various periods. After cells had bound to the various substrates, the wall shear stress was increased stepwise every 5 s (by a programmed set of flow rates delivered by the syringe pump) to 40 dyn/cm^2. At the end of each 5-s interval of the increase in shear stress, the number of cells that remained bound was expressed relative to the number of cells that accumulated on VCAM-1 in flow or that bound on FN in stasis. The contribution of cells rolling into the observation field from upstream fields was minimized by locating the field at the upstream edge of the spot of adsorbed ligands. Reduction in the number of cells remaining bound in the field at elevated shear stresses was due both to cell detachment from the substrate and to cells rolling out of the field of view without being detained. During the detachment experiments, cell fractions that rolled, i.e., cells that moved ≥0.5 μm/s for at least 3 s, were assessed at selected shear stresses. Unless otherwise indicated, the assays were performed at room temperature, which minimized spontaneous cell activation or change in shape. Rolling velocities were determined for cells tethered under flow by analysis of cell displacements over 5- to 10-s intervals, typically 2–3 s after the initial tethering event.

Pretreatments of cells and substrates for the flow experiments For Ab inhibition studies, cells (10^7/ml) were preincubated (5 min, 4°C) in H/H medium with VLA-4-blocking mAb (20 μg/ml). The cells were diluted 1/10 into binding medium (H/H medium containing 1 mM Ca^{2+} and 1 mM Mg^{2+}) without washing out the Ab, and the suspension was perfused into the flow chamber. For metabolic inhibition experiments, cells (10^7/ml) were treated (10 min, 22°C) with 0.1% NaN_3 (azide) and 50 mM 2-deoxyglucose (2-DOG) in H/H medium and then diluted (1/10) with binding medium containing 0.05% azide and 25 mM 2-DOG. To interfere with the integrity of their actin cytoskeleton, leukocytes were suspended in binding medium containing 20 μM cytochalasin B for 10 min at 24°C, before being perfused, unwashed, through the flow chamber. To study peptide inhibition, cells were suspended in binding medium with or without 0.1–1 mM of the octapeptide EILDVPST or its control analogue EIDVLPST and were incubated for 5 min at 24°C before perfusion through the flow chamber. The effect of cell activation on cell binding to substrates was assessed by preincubation (3 min, 24°C) in binding medium containing either PMA (100 ng/ml) or the β1 activating mAb TS2/16 (5 μg/ml); the cells were not washed before perfusion through the chamber. For assessment of cation-induced integrin activation, H/H medium containing 0.2 mM Mn^{2+}, rather than binding medium, was used during cell perfusion through the chamber.

The functional activity of VCAM-1 on the ligand-coated plates used in the flow chamber was blocked by pretreatment with the anti-VCAM-1 mAb 4B9. Medium containing the mAb (20 μg/ml) was perfused (for 20 min) through the flow chamber. mAb (5 μg/ml) was also added to the binding medium in which cells were perfused into the chamber. Background adhesive interactions of the cells were determined by analyzing cell tethering on plates coated with HSA (20 mg/ml). The adhesive interactions of differently pretreated cells were assessed on identical microscopic fields; this ensured that variance in distribution of the immobilized ligands in each test field could not affect the adhesive properties of the compared cells. Both VCAM-1 and FN remained functionally intact throughout the experiment, as verified by comparing the behavior of control cells in multiple tests performed either at the start or at the end of each series of experiments.

Statistical analysis Two-tailed Student’s t test was used, where indicated, to determine the level of significance of differences in mean values of paired experimental groups.

Results Inducible and constitutive VLA-4 adhesiveness is impaired in an activation mutant of Jurkat T cells The VLA-4-mediated adhesion to VCAM-1 of wt and activation mutant Jurkat cells termed 11.1 was characterized by controlled detachment assays using a parallel plate flow chamber. The shear resistance developed by wt and mutant cells briefly adhered to VCAM-1 substrates was analyzed by subjecting the adherent cells to incremental increases in shear stress. PMA treatment significantly increased the resistance to detachment of wt Jurkat cells from VCAM-1, but only slightly augmented the shear resistance of the mutant cells (Fig. 1, A and B). Mn^{2+} dramatically enhanced the resistance to detachment from VCAM-1 of both wt and activation mutant cells (Fig. 1, A and B), indicating that the ability of VLA-4 to undergo cation-induced activation remained intact on the mutant cells. Interestingly, the adhesions to VCAM-1 of unactivated Jurkat cells were more shear resistant than those of the unactivated mutant cells (Fig. 1). Both wt and mutant cells expressed comparable levels of α4 and β1 integrin chains on their surface, as previously shown (22). Thus, besides the inability of the mutant cells to up-regulate their VLA-4 adhesiveness by exogenous cellular activation, VLA-4 on these cells exhibited a defect in constitutive adhesive activity.

The strength of VLA-4-mediated adhesion spontaneously developed by resting mutant and wt Jurkat cells was analyzed on plates coated with a medium concentration of VCAM-1 (Fig. 2A). The shear resistance of the resting mutant cells or of energy-depleted Jurkat cells was much lower than that of the wt Jurkat resistance on both high and low VCAM-1 densities (Fig. 2A and data not shown). The shear resistance of wt, mutant, and energy-depleted cells dropped rapidly as the VCAM-1 concentration adsorbed on the substrate was reduced (data not shown). This higher resistance of wt T cells occurred regardless of whether they had accumulated under low shear flow or they had adhered to VCAM-1 in stasis. Furthermore, prolonged accumulation on VCAM-1 did not rescue the adhesion deficiency of the mutant cells, suggesting that it did not result from a slower kinetics of adhesion strengthening of the
mutant cells (data not shown). The strong VLA-4-mediated adhesion of resting wt Jurkat on VCAM-1 was associated with reduced rolling relative to that of resting mutant cells. Although the majority of mutant cells could roll on the VCAM-1 substrate at shear stresses >0.5 dyn/cm², most wt Jurkat cells remained bound and stationary on the VCAM-1 substrate under similar conditions (Fig. 2B). Surprisingly, energy-depleted wt Jurkat cells also exhibited persistent rolling adhesions on VCAM-1. Similar patterns of increased mutant rolling were observed on VCAM-1-transfected CHO cells and on cytokine-stimulated VCAM-1-expressing endothelial cell monolayers (data not shown).

Affinity states and surface distribution of VLA-4 on resting wt and activation mutant Jurkat cells

The preferential presentation of α4 integrins on lymphocyte microvilli (8, 37) has been suggested to facilitate leukocyte tethering under high shear flow to endothelial counter-receptors. Therefore, the VLA-4 distribution on mutant and wt cells was compared by transmission electron microscopy. The cells were prefixed in the presence of physiological concentrations of Ca²⁺ and Mg²⁺, and immunogold staining of α4 was performed with mAb directed against epitope distal from the ligand binding site. The surface topology was similar with regard to microvilli number and dimensions of the wt and mutant cells (Fig. 3). VLA-4 was localized predominantly to the microvilli in both cell types. A small amount of the α4 molecules of both cell types was present in small clusters that consisted of two or more gold particles. Thus, the deficient VLA-4 adhesiveness of the mutant could not be due to an altered surface distribution of VLA-4.

Freely flowing cells can be captured in shear flow by immobilized mAbs specific for cell surface Ags (38, 39). Capture assays, performed on immobilized anti-L-selectin mAb, can functionally differentiate between L-selectin located on microvilli and L-selectin located on the cell body. Therefore, a similar assay was used to compare the surface availability of VLA-4 on wt and 11.1 mutant Jurkat cells. At all the shear stresses tested, both cell types were captured at similar rates on a substrate coated with an anti-VLA-4 mAb (Fig. 4B and data not shown), suggesting that VLA-4 on both wt and mutant cells is accessible to the same extent for interaction with an immobilized VLA-4-specific mAb under shear flow. In contrast, at shear stresses >1.2 dyn/cm², energy-depleted wt Jurkat cells were captured less efficiently than untreated cells by the immobilized mAb (Fig. 4A). Cell capture by the anti-VLA-4 mAb was abrogated by cytochalasin B treatment (Fig. 4A). Since at elevated shear flow, cell capture becomes more sensitive to the surface accessibility of the tethering receptor (38, 40), these results suggested that the surface accessibility of VLA-4 was partially reduced by energy depletion of wt cells, but was fully retained on the 11.1 mutant.

The reduced adhesiveness of VLA-4 on these cells could be the result of an altered intrinsic affinity of the integrin to ligand. VLA-4 recognizes an LDV-containing sequence on the CS-1 domain of FN (41). This sequence is homologous and isosteric with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the VLA-4 binding site of FN (41). This sequence is homologous and isosteric with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the VLA-4 binding site of FN (41). This sequence is homologous and isosteric with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the VLA-4 binding site of FN (41). This sequence is homologous and isosteric with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the VLA-4 binding site of FN (41). This sequence is homologous and isosteric with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the VLA-4 binding site of FN (41). This sequence is homologous and isosteric with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the VLA-4 binding site of FN (41).
significantly weaker LDV dose-response of LIBS staining was observed in these cells (Fig. 4B). In energy-depleted wt cells, LIBS induction by LDV was almost nonexistent (Fig. 4B). These results indicate that VLA-4 on resting 1.1 mutant and energy-depleted wt cells recognize the LDV peptide with a considerably lower affinity than VLA-4 on resting wt cells.

Tethering, rolling, and adhesion strengthening of wt and activation mutant Jurkat cells on VCAM-1 and FN

VLA-4 adhesions to VCAM-1 in shear flow can be divided into three distinct adhesive stages: tethering, rolling, and spontaneous cell arrest. Spontaneous cell arrest is established either immediately upon the initial cell tethering or after a period of rolling on the ligand. To learn about the molecular basis of these distinct processes, different types of adhesive interactions between wt and mutant Jurkat cells with different densities of VCAM-1 were examined at representative physiological shear stresses. Cells were individually tracked for the first 10 s after their initial tethering to the substrate. Tethers were categorized as transient, rolling, rolling followed by spontaneous arrests, and immediate arrests. The spontaneous arrest category included rolling cells that spontaneously arrested on VCAM-1 within less than 10 s. Cells that immediately arrested upon tethering and remained stationarily bound to ligand for at least 20 s were assigned to the arrest category.

The accumulation of wt Jurkat cells at a medium shear stress on a high density VCAM-1 field (coated at 2 μg/ml) was 3 times that of the 1.1 mutant (58 ± 6 vs 20 ± 4 cells/field.min) and of energy-depleted wt cells (22 ± 6 cells/field.min). The vast majority of the tethered mutant and energy-depleted cells continued to roll after tethering on VCAM-1. In contrast, as much as 40% of the wt Jurkat cells immediately arrested upon tethering and remained stationarily bound to ligand for at least 20 s were assigned to the arrest category.

The accumulation of wt Jurkat cells at a medium shear stress on a high density VCAM-1 field (coated at 2 μg/ml) was 3 times that of the 1.1 mutant (58 ± 10 vs 20 ± 4 cells/field.min) and of energy-depleted wt cells (22 ± 6 cells/field.min). The vast majority of the tethered mutant and energy-depleted cells continued to roll after tethering on VCAM-1. In contrast, as much as 40% of the wt Jurkat cells immediately arrested upon tethering and remained stationarily bound to ligand in shear flow (Fig. 5A). Another 25% of the tethered wt cells arrested spontaneously within <10 s following tethering. The fraction of immediately or spontaneously arresting T cells diminished on substrates coated at lower coating concentrations even when considerable tethering and rolling were still observed, consistent with previous findings that spontaneous arrest of lymphocytes on VCAM-1 increases with VCAM-1 density (43). When the VCAM-1 substrate was blocked by 4B9 mAb, which is directed against domain 1 of VCAM-1, no T cell tethers occurred (data not shown). This indicates that the tethers were VCAM-1 specific and that domain 4 on VCAM-1 is not involved in VLA-4-mediated tethering under flow. When tethering was measured at a high shear stress (2.5 dyn/cm²), its frequency decreased similarly for both wt and 1.1 mutant cells, the tethers became more labile, and most wt
cells failed to immediately arrest on VCAM-1, but, rather, arrested after short rolling periods (Fig. 5B). Notably, wt Jurkat and its activation mutant tethered to VCAM-1 at identical rates even at these extreme shear stresses. Essentially none of the mutant cells spontaneously arrested on VCAM-1 (Fig. 5B), but they could do so in the presence of the VLA-4-activating reagents mAb TS2/16 or Mn²⁺ (data not shown). High fractions of wt, but not mutant, cells spontaneously arrested in a VLA-4-dependent fashion on a monolayer of VCAM-1-transfected CHO cells, although similar fractions of wt and 11.1 mutant cells could initiate tethers on the cell-based VCAM-1 (data not shown). When VCAM-1 was coated below a threshold concentration of 0.1 μg/ml, it could no longer support rolling or arrests. Under these conditions, wt, 11.1 mutant, and energy-depleted Jurkat cells tethered at identical efficiencies to low density VCAM-1 (Fig. 5C).

Similar to our findings with the Jurkat T cell line (Fig. 5), energy depletion of PBL reduced their accumulation on high density VCAM-1 substrates at physiological shear stresses by 55–60%. This reduction was accompanied by a destabilization of T cell resistance to detachment at elevated shear stresses (data not shown). As seen with the Jurkat T cell line, a substantial number of PBL immediately arrested shortly after tethering on high density VCAM-1 in flow, and that arrest required cellular energy (Fig. 6A). Higher VCAM-1 densities were required to immediately arrest tethered PBL than to arrest Jurkat cells (Figs. 5A and 6A). Of the energy-depleted PBL tethered to high density VCAM-1, almost all lymphocytes failed to arrest and continued to roll on VCAM-1 at lower densities.
as untreated cells to FN40, similar to their near-identical tethering rates to low density VCAM-1 (Fig. 5C). Nevertheless, the majority of tethers by wt Jurkat cells, but not those of the 11.1 mutant and energy-depleted wt cells, were followed by immediate cell arrest on the ligand. Thus, the initial VLA-4-mediated tethering on both VCAM-1 and FN CS-1 in shear flow is not impaired in the mutant or by energy depletion of wt cells, but the spontaneous translation of tethers into firm arrests is.

Involved of high affinity VLA-4 in spontaneous arrests of T cells on VCAM-1

The ability of wt T cells to spontaneously arrest upon tethering to VCAM-1 in shear flow (Figs. 5 and 6) and the presence of high affinity VLA-4 on these T cells suggested that this high affinity VLA-4 form is essential for rapid development of adhesion strengthening. To monitor the function of different affinity states in cells interacting with surface-bound ligand under flow, we developed a peptide-blocking assay in which high affinity VLA-4 states become preferentially occupied by soluble LDV-containing peptide. To verify the specificity of the assay for high affinity states, either low or high affinity VLA-4 states were artificially induced on Jurkat cells in medium containing either Ca$^{2+}$ or Mg$^{2+}$, respectively. Although at stasis, VLA-4 supported weaker adhesions in the presence of Ca$^{2+}$ alone on both VCAM-1 and FN (data not shown), VLA-4 tethering to VCAM-1 in shear flow was comparable in the presence of either cation (Fig. 8A). This suggested that both low affinity (Ca$^{2+}$-dependent) and high affinity (Mg$^{2+}$-dependent) VLA-4 tether T cells to VCAM-1 under flow with comparable efficiencies. In agreement with our assumption, the LDV peptide, but not the control DVL-containing peptide, could block tethering of wt Jurkat cells to low density VCAM-1 in the presence of Mg$^{2+}$, but not in the presence of Ca$^{2+}$ (Fig. 8A). Accordingly, cell preincubation with mAb TS2/16, a β1 integrin affinity-stimulating mAb, rendered VLA-4-mediated tethering to VCAM-1 more susceptible to inhibition by the LDV peptide (data not shown). The LDV peptide inhibition of tethering was dose dependent, with a maximum reached at 0.5 mM (data not shown). These collective results confirmed our hypothesis that LDV can selectively block tethers supported by high affinity VLA-4 states. In physiological medium containing equimolar levels of both Ca$^{2+}$ and Mg$^{2+}$, inhibition by the LDV peptide of VLA-4-mediated tethering of wt Jurkat cells to low density VCAM-1 was half that obtained in medium containing Mg$^{2+}$ alone (25–30 vs 50%; Fig. 8B). In contrast, VLA-4-mediated tethering of the 11.1 activation mutant as well as that of energy-depleted wt Jurkat cells to VCAM-1 were absolutely insensitive to inhibition by the LDV peptide in physiological medium (Fig. 8B).

The extent to which the LDV-sensitive VLA-4 states naturally occurring on wt Jurkat cells contribute to the various categories of tethers on high density VCAM-1 was next examined. The wt and mutant Jurkat cells were perfused on VCAM-1 in binding medium alone or with the LDV or DVL octapeptides. Each cell type exhibited comparable tethering frequencies with similar fractions of the different categories of tethers in medium alone and in the presence of the control DVL peptide (Fig. 9A). In medium alone or in the presence of the control peptide, 34–42% of initially tethered wt cells arrested immediately upon tethering to VCAM-1, and an additional 32–35% of tethered cells were arrested after a short rolling period (Fig. 9A). In the presence of saturating levels of the LDV peptide, on the other hand, total tethering of wt Jurkat cells to VCAM-1 was reduced by about 25% (Fig. 9A). Strikingly, the remaining wt Jurkat cells tethered to VCAM-1 could not immediately arrest on the VLA-4 ligand, but continued to roll on it (Fig. 9A), indicating that the LDV-sensitive, high affinity VLA-4 subset...
supports all immediate arrests of wt cells tethered to VCAM-1 under shear flow. The LDV peptide also partially interfered with the ability of tethered cells to spontaneously arrest during rolling on VCAM-1, which suggests that this category of tethers is mediated by a heterogeneous VLA-4 subset with overall lower affinity than that of the VLA-4 subset responsible for immediate cell arrest on VCAM-1.

In contrast, transiently tethered wt cells and cells that tethered and rolled on VCAM-1 without spontaneously arresting on the ligand were completely insensitive to the LDV peptide even at

FIGURE 7. Tethering and adhesion strengthening of wt and 11.1 mutant Jurkat on FN and its VLA-4 binding fragment. Adhesion of wt and 11.1 mutant Jurkat cells to FN (A) and to the VLA-4-binding CS-1 fragment of FN, FN40 (B), in stasis is shown. Cells were allowed to adhere for 10 min at 37°C to FN (coated at 10 μg/ml; A) or for 30 s at 24°C to the CS-1 fragment (FN40, coated at 5 μg/ml; B) before being subjected to incremented shear stresses. The mean ± range of data collected in two fields of view are depicted. C, Tethering of wt, energy-depleted wt, and 11.1 mutant Jurkat cells and of the CS-1 fragment (coated at 5 μg/ml) at a subphysiological shear stress of 0.3 dyn/cm². Data from a single representative field are depicted. The experiments described in A–C are representative of at least three independent experiments.

FIGURE 8. Effect of LDV peptide on VLA-4-mediated tethering of Jurkat cells to low density VCAM-1 in the presence of different divalent cations. A, Tethering of wt Jurkat cells to VCAM-1 (coated at 0.1 μg/ml) in medium containing either 2 mM Ca²⁺ or 2 mM Mg²⁺ at a shear stress of 0.5 dyn/cm². Cells were prewashed with EDTA and resuspended in the indicated medium with or without 0.5 mM of the indicated peptides for 5 min at 24°C before perfusion (in the continued presence of the peptides) over VCAM-1. B, Effects of LDV and DVL peptides on the transient tethering of wt, 11.1 mutant, and energy-depleted wt Jurkat cells to low density VCAM-1. Tethering rates were determined at a shear stress of 0.5 dyn/cm² in standard binding medium containing both Ca²⁺ and Mg²⁺. Data depicted are the average from two fields of view from one experiment representative of four. *, p < 0.05; **, p < 0.07.
saturating concentrations (Fig. 9A). Another soluble ligand to VLA-4, VCAM-1-Ig fusion protein, at saturating levels not only blocked all immediate arrests, but also completely interfered with the ability of tethered cells to spontaneously arrest during rolling on VCAM-1 (Fig. 9B). VCAM-1-Ig also reduced the total tethering of wt cells by 70%. The residual transient tethers and tethers followed by rolling adhesions were still completely insensitive to sVCAM-1 (Fig. 9B). Similar selectivity of inhibition of cell arrest, but not of rolling adhesions, by soluble LDV and VCAM-1-Ig was observed with Jurkat cells perfused over substrates coated with both VCAM-1 and the endothelial L-selectin ligand, peripheral node addressin (44) (data not shown). The mean ± error range of values from two fields of view are depicted. A representative experiment of three is shown.

By sharp contrast to wt cells, the tethering of the 11.1 mutant Jurkat and of energy-depleted wt Jurkat cells to high density VCAM-1 was not susceptible to inhibition by the LDV peptide (Fig. 9A). Energy-depleted wt Jurkat cells initiated fewer total tethers than wt or 11.1 mutant cells on VCAM-1 (Fig. 9A) at physiological shear stresses, consistent with previous findings (Figs. 4–6 and 8). As shown above (Fig. 5), both mutant and energy-depleted cells established efficient rolling, but usually failed to arrest upon tethering to high density VCAM-1. The small fraction of mutant cells that did arrest after a short period of rolling was insensitive to sVCAM-1-Ig (Fig. 9B). With wt cells, neither LDV nor VCAM-1-Ig interfered with the rolling or transient tether categories of the 11.1 mutant or the energy-depleted wt cells. The LDV peptide did not affect the rolling velocities of the wt Jurkat fraction that initiated rolling after tethering to VCAM-1 (Fig. 9B), further indicating that rolling adhesions are mediated primarily by low affinity, LDV-insensitive VLA-4 states. Although the rolling dynamics of the 11.1 mutant and those of energy-depleted Jurkat cells were as insensitive to the presence of LDV peptide as those of wt cells, both types of cells rolled slightly faster than wt cells (Fig. 9B). High affinity, LDV-sensitive states of VLA-4 also contributed to the development of high resistance of wt Jurkat cells to detachment by elevated shear forces from VCAM-1; in the presence of the LDV peptide, wt cells detached more readily from VCAM-1 (data not shown). Similar to Jurkat cells, peripheral blood CD4+ T cells...
could spontaneously arrest on high density VCAM-1, either immediately upon tethering or after short periods of rolling. About half the individual PBL examined could establish either immediate or rolling-associated arrests after tethering to high density VCAM-1 (Fig. 9D). As with the Jurkat cells, all immediate arrests and most of the rolling-associated arrests could be inhibited by saturating levels of the LDV peptide, but not by the control DVL peptide (Fig. 9D). Rolling and transient tethers of PBL on VCAM-1 were insensitive to the LDV peptide. These findings indicate that low affinity VLA-4 states preferentially mediate the transient tethering and rolling of PBL that fail to spontaneously arrest on VCAM-1. The high affinity VLA-4 states of PBL preferentially support all types of spontaneous arrests on VCAM-1 in a homologous manner to the high affinity VLA-4 subsets of wt Jurkat cells.

Discussion

The interaction of VLA-4 integrin with VCAM-1 can support all the adhesive steps required for arrest of lymphocytes on inflamed vascular endothelia in the absence of exogenous chemokines (7, 8, 34). VLA-4, besides supporting tethering and rolling of unstimulated T lymphocytes on immobilized VCAM-1 under shear flow conditions, also mediates spontaneous lymphocyte arrest on high density VCAM-1, either immediately or soon after rolling adhesions are established on the ligand, thus bypassing chemotactant stimulatory steps (7). The molecular basis for this versatility of adhesive functions mediated by a single receptor:ligand pair may reside in the multiple activation states this integrin can adopt. The activation states of VLA-4, which are primarily defined by their differential avidity to immobilized ligand, not only vary according to the cell type on which VLA-4 is expressed, but also are dynamically regulated by various inside-out signals (45–49). In the present study a quantitative approach was developed to examine the contribution of VLA-4 affinity to the initial cell tethering to immobilized ligand under dynamic conditions of flow, to the establishment and propagation of rolling adhesions on VCAM-1, and to the arrest and development of shear-resistant cell adhesion on the endothelial ligand. Along with PBL, the T lymphoblastoid Jurkat line was used as a model for studying VLA-4 integrin function in CD4+ T cells. VLA-4 adhesiveness under shear flow was compared in wt Jurkat cells and in a mutant with major defects in activation-dependent regulation of β1 integrin function (22).

Although the activation mutant was originally selected for its inability to respond to exogenous signals associated with TCR- and PKC-dependent signaling (22), it also exhibits impaired VLA-4 adhesiveness in its resting state despite expressing VLA-4 at levels comparable to those of wt Jurkat cells. The mutant could tether efficiently to different VLA-4 ligands and establish rolling adhesions on VCAM-1 under shear flow, but it failed to arrest on ligand and could not develop firm adhesion (Figs. 2 and 5). Immunolocalization of VLA-4 by electron microscopy indicated similar distribution of the integrin on the surfaces of wt and mutant cells. VLA-4 on mutant cells was as available for binding ligand as VLA-4 on wt cells, as indicated by the similar efficiencies by which wt and mutant cells could tether to a substrate-bound anti-VLA-4 mAb under shear flow (Fig. 4). Moreover, in the presence of nonphysiologically VLA-4 agonists, such as Mn2+ or the affinity-stimulating mAb TS2/16, which artificially stabilized the mutant’s VLA-4 at high affinity state, the mutant rapidly developed firm adhesions and spread on VCAM-1. This observation not only suggested that the VLA-4 in the mutant remained structurally intact with respect to its ability to acquire high affinity conformation if properly activated, but it also indicated that the cytoskeletal associations of VLA-4 were not impaired in the mutant. We therefore reasoned that the reduced adhesiveness of VLA-4 in the mutant, in both its unstimulated and stimulated states, may result from an inability of the VLA-4 on the mutant to acquire high affinity to ligand. VLA-4 affinity to ligand is very low in physiological medium and cannot be monitored by direct cell binding assays using monovalent physiological ligands of VLA-4, because such ligands are readily washed out from the cell surface during these assays. The affinity to LDV of the high affinity VLA-4 subset characterized here on wt Jurkat cells was enhanced by at least 10-fold in the presence of Mn2+ (50) (data not shown). Even under these conditions, direct binding of sVCAM-1 or CS-1 fragments could not be assessed. VLA-4 affinity states were therefore assessed by their ability to express a ligand-induced epitope (LIBS) in the presence of a soluble LDV-containing peptide or VCAM-1 (50). This approach identified a small subset of high affinity VLA-4 receptors that are constitutively expressed on unstimulated wt Jurkat cells, but are depleted in the mutant cells. Strikingly, the ability of VLA-4 to support cell tethering to immobilized VCAM-1 or FN CS-1 did not require the presence of the high affinity subset, as suggested by the identical tethering rates of both the activation mutant and wt Jurkat cells observed on both ligands. The wt Jurkat tethering to VCAM-1 was also comparable in the presence of Mg2+-depleted Ca2+-containing medium, which is known to suppress VLA-4 affinity, and as in high affinity-inducing binding medium that contained Mg2+ only (45). These results further suggest that low affinity VLA-4 and high affinity VLA-4 support similar cell tethers on ligand under the shear flow conditions tested.

The distribution of LDV-induced LIBS epitopes on Jurkat cells suggested that VLA-4 occurs in heterogeneous affinity states on resting T cells. Heterogeneity of integrin affinity states on individual leukocytes and fibroblasts has previously been reported (18, 51, 52). We assumed that high affinity VLA-4 states could be distinguished from low affinity states by their preferential occupancies of and inhibition by a soluble ligand, as was previously shown with other integrins (18, 19). Indeed, the LDV peptide could selectively inhibit tethers mediated by high affinity VLA-4, artificially induced by Mg2+ or by an integrin-activating mAb. The peptide failed to inhibit tethers supported by low affinity VLA-4, exclusively expressed on energy-depleted T cells or on wt cells in Mg2+-depleted, Ca2+-containing medium (Fig. 8). This differential susceptibility of high and low affinity VLA-4 to inhibition by soluble ligand allowed us to dissect the contribution of each of these states to initial tethering, rolling, and spontaneous arrests of different T cells on VCAM-1-coated substrates under shear flow. Using this approach, we determined that the presence of high affinity VLA-4 is mandatory for wt T cells to rapidly develop spontaneous arrests upon tethering to high density VCAM-1; selective inhibition by soluble ligand eliminated the ability of wt cells to arrest on VCAM-1 immediately after tethering to the ligand under shear flow. In contrast, the peptide did not block any transient tethers or rolling adhesions initiated by wt cells on high density VCAM-1, suggesting that low affinity VLA-4 states are exclusively associated with these labile adhesive interactions between flowing wt cells and surface-bound VCAM-1. Similarly, high affinity (LDV-sensitive) VLA-4 on freshly isolated PBL was indispensible for their spontaneous adhesion strengthening on high density VCAM-1, whereas low affinity (LDV-insensitive) VLA-4 states supported rolling adhesions on the ligand. In both Jurkat cells and PBL, the overall number of initial tethers to VCAM-1, in addition to the extent of spontaneously arrested tethers, was reduced in the presence of the LDV peptide. This indicates that once bound to a high affinity VLA-4 receptor, the peptide not only blocked the ability of the integrin to support cellular arrests on
VCAM-1, but also interfered with its ability to promote initial cell tethering (Fig. 9).

A more complex scheme of inhibition by soluble LDV peptide was observed on the spontaneous arrests of wt Jurkat cells, which developed during cell rolling on VCAM-1, rather than immediately after cell tethering to ligand. Although a portion of these spontaneous arrests were insensitive to inhibition by the LDV-containing peptide, they could be still blocked by sVCAM-1-Ig. An alkaline phosphatase-tagged VCAM-1-Ig binds VLA-4 on Jurkat cells with an $IC_{50}$ of 3 nM in physiological medium (R. Lobb, unpublished observations), whereas the LDV octapeptide used here binds VLA-4 with an $IC_{50}$ of 100 μM (Fig. 4). This suggests the presence on Jurkat cells of intermediate affinity states that are not inhibited by an LDV-containing peptide but are inhibited by the more potent ligand, VCAM-1-Ig. Taken together, these results suggest that the spontaneous arrests developed by wt Jurkat rolling on VCAM-1 appear to be mediated in part by high affinity and in part by intermediate affinity VLA-4. Although a potent VLA-4 ligand, VCAM-1-Ig, did not affect the fractions of wt Jurkat cells that established rolling or transient tethering to VCAM-1 even at a concentration 20-fold higher than its $IC_{50}$ (Fig. 9B). This is a further indication that rolling and transient tethers are supported by extremely low affinity states of VLA-4, which are not occupied even in the presence of saturating levels of sVCAM-1. Similarly, in PBL, spontaneous arrests, but none of the transient tethers or rolling adhesions, were inhibited by saturating levels of ligand (Fig. 9). This finding suggests that high affinity VLA-4 states constitutively expressed on circulating T lymphocytes are mandatory for the ability of these lymphocytes to spontaneously arrest on VCAM-1, but are dispensable for the rolling adhesions of these lymphocytes on the vascular VLA-4 ligand.

A flowing T cell may form a primary tether to endothelial VCAM-1 through either its low affinity or its high affinity VLA-4 receptors. In the first case, low affinity interactions between VLA-4 and VCAM-1 would result in rolling adhesions until high affinity VLA-4, preexistent on the rolling cell, becomes available for binding immobilized VCAM-1 and instantaneously arrests the rolling cell. In the second case, an initial tether is mediated by high affinity VLA-4, and the tether bond is sufficiently strong to immediately arrest the tethered T cell under flow. Consistent with such a stochastic mechanism, prolonged periods of rolling were observed to increase the fractions of wt Jurkat or PBL that developed spontaneous arrests on VCAM-1. Interestingly, a general tyrosine phosphorylation inhibitor, genistein, known to interfere with VLA-4 outside-in signaling (53), did not reduce these fractions (data not shown). These results suggest that during rolling on VCAM-1, new high affinity states are not induced on the adherent cells, nor does VLA-4 avidity to the immobilized ligand increase through outside-in signaling. Nevertheless, a novel linkage between normal leukocyte functions within noninflamed tissues (3). Acknowledgments

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References


The pre-existent expression on T cells of high affinity VLA-4 may be of general significance, because VLA-4 adhesiveness is generally up-regulated by inside-out signaling pathways, which do not increase the intrinsic affinity of integrin for ligand (20, 32, 56). High affinity VLA-4 could support rapid lymphocyte arrest on inflamed vessels that express high levels of VCAM-1 (57) before or along with T cell activation by endothelium-displayed chemokines (14, 58). VCAM-1 is highly expressed on inflamed brain endothelium in models of experimental allergic encephalomyelitis (3), and in vivo blocking experiments indicate an exclusive role for VLA-4:VCAM-1 interactions in the recruitment of T lymphocytes to inflamed microvasculature of the central nervous system (57). In such settings and in other inflammatory sites that express high levels of VCAM-1, the application of soluble VLA-4 ligands or of reagents that elevate sVCAM-1 levels in serum (59) is expected to selectively block small subsets of high affinity VLA-4, leaving the majority of VLA-4 intact. These approaches may afford therapeutic advantages over VLA-4 blocking by mAbs, which interferes with normal leukocyte functions within noninflamed tissues (3).


