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### Article

Leukocytes extravasate from the blood in response to physiologic or pathologic demands by means of complementary ligand interactions between leukocytes and endothelial cells. The multistep model of leukocyte extravasation involves an initial transient interaction ("rolling" adhesion), followed by secondary (firm) adhesion. We recently showed that binding of CD44 on activated T lymphocytes to endothelial hyaluronan (HA) mediates a primary adhesive interaction under shear stress, permitting extravasation at sites of inflammation. The mechanism for subsequent firm adhesion has not been elucidated. Here we demonstrate that the integrin VLA-4 is used in secondary adhesion after CD44-mediated primary adhesion of human and mouse T cells *in vitro*, and by mouse T cells in an *in vivo* model. We show that clonal cell lines and polyclonally activated normal T cells roll under physiologic shear forces on hyaluronate and require VCAM-1, but not ICAM-1, as ligand for subsequent firm adhesion. This firm adhesion is also VLA-4 dependent, as shown by antibody inhibition. Moreover, *in vivo* short-term homing experiments in a model dependent on CD44 and HA demonstrate that superantigen-activated T cells require VLA-4, but not LFA-1, for entry into an inflamed peritoneal site. Thus, extravasation of activated T cells initiated by CD44 binding to HA depends upon VLA-4-mediated firm adhesion, which may explain the frequent association of these adhesion receptors with diverse chronic inflammatory processes.

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# The CD44-initiated pathway of T-cell extravasation uses VLA-4 but not LFA-1 for firm adhesion

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Leukocytes extravasate from the blood in response to physiologic or pathologic demands by means of complementary ligand interactions between leukocytes and endothelial cells. The multistep model of leukocyte extravasation involves an initial transient interaction (“rolling” adhesion), followed by secondary (firm) adhesion. We recently showed that binding of CD44 on activated T lymphocytes to endothelial hyaluronan (HA) mediates a primary adhesive interaction under shear stress, permitting extravasation at sites of inflammation. The mechanism for subsequent firm adhesion has not been elucidated. Here we demonstrate that the integrin VLA-4 is used in secondary adhesion after CD44-mediated primary adhesion of human and mouse T cells *in vitro*, and by mouse T cells in an *in vivo* model. We show that clonal cell lines and polyclonally activated normal T cells roll under physiologic shear forces on hyaluronate and require VCAM-1, but not ICAM-1, as ligand for subsequent firm adhesion. This firm adhesion is also VLA-4 dependent, as shown by antibody inhibition. Moreover, *in vivo* short-term homing experiments in a model dependent on CD44 and HA demonstrate that superantigen-activated T cells require VLA-4, but not LFA-1, for entry into an inflamed peritoneal site. Thus, extravasation of activated T cells initiated by CD44 binding to HA depends upon VLA-4-mediated firm adhesion, which may explain the frequent association of these adhesion receptors with diverse chronic inflammatory processes.

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## Introduction

Coordinated regulation and appropriate site-specific targeting of leukocyte extravasation results from various types of adhesion receptors acting in sequential fashion, allowing directed leukocyte exit from the blood into diverse tissues within the organism (1, 2). Thus, the elucidation of molecules mediating the extravasation of leukocytes into targeted sites is central to the understanding of the complex regulation of the host response to inflammation. Several families of adhesion receptors are thought to be essential in this multistep model of extravasation for both nonspecific effector cells and antigen-specific lymphocytes. The initial contact with and primary adhesion (“rolling”) of leukocytes on vascular endothelium has generally been attributed to the engagement of members of the selectin family by their carbohydrate ligands. Subsequent secondary (firm) adhesion is largely due to heterodimeric integrins interacting with their endothelial cell ligands, members of the immunoglobulin gene superfamily (3, 4). After arrest, leukocytes migrate through the endothelial and basement membrane barriers into underlying tissues.

Tissue-specific migration pathways of effector and memory T-lymphocyte subsets have been shown to be distinct, and in part attributable to adhesion receptors that they express (5–9). Our laboratory has been

engaged in the description and characterization of a pathway of extravasation initiated by CD44 on lymphocytes activated to bind its major ligand, hyaluronan (HA). CD44 interactions with HA expressed on endothelial cells permit lymphocyte rolling under shear conditions simulating physiologic flow (10). Although the mechanism by which CD44 becomes activated to bind HA has not yet been completely elucidated, it has become clear that stimulation of normal T lymphocytes *in vitro* or *in vivo* through the T-cell receptor induces the activated form of CD44 and attendant primary adhesion on endothelium or HA substrate (11–13). These observations have established the HA-binding form of CD44 as an early activation marker on T cells after T-cell receptor stimulation, and support a role for this interaction during the course of an immune response. Moreover, CD44/HA interactions have been shown to be required for extravasation of superantigen-stimulated T cells into an inflamed peritoneal site in a mouse model (12). CD44 has been prominently associated with human arthritis and with a model of collagen-induced murine arthritis (14–17), and more recently with a model of multiple sclerosis, experimental allergic encephalomyelitis (18). CD44 interactions with HA are also thought to be important in allogeneic graft rejection (19, 20). We have proposed that this CD44-initiated pathway is used primarily by activated T cells for

gress into a variety of inflamed tissues. Consistent with this suggestion, the presence of circulating T cells bearing the activated form of CD44 that enables HA-dependent rolling correlates highly with exacerbation of major autoimmune disease in humans (15). Evidence suggests that HA binding through CD44 can be regulated through mechanisms such as glycosylation and a variety of other posttranslational modifications (21–25), but the particular molecular basis for CD44 activation of this subset of T cells has not been clarified.

The predominant interactions observed in laminar flow assays between activated T cells bearing CD44 and an HA substrate have been those of primary adhesion. For physiologic relevance, these interactions would require subsequent firm adhesion and arrest to achieve extravasation, as predicted by our model and by *in vivo* evidence (12). Because secondary adhesion preceded by selectin-mediated primary adhesion is generally mediated by integrins, we chose to examine the 2 integrins that are most prominently expressed on most circulating T cells – VLA-4 ( $\alpha 4\beta 1$ ) and LFA-1 ( $\alpha L\beta 2$ ) – and their ligands as candidates for secondary adhesion subsequent to CD44-mediated rolling. We report that in these studies, VLA-4 is the major integrin used in this pathway both *in vitro* and in an *in vivo* model, whereas LFA-1 contributes little to this process.

## Methods

**Reagents.** PMA and staphylococcal enterotoxin B (SEB) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Rooster comb sodium hyaluronate was from Fisher Scientific Co. (Pittsburgh, Pennsylvania, USA). Ionomycin was from Calbiochem-Novabiochem Corp. (La Jolla, California, USA), and 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA) came from Molecular Probes Inc. (Eugene, Oregon, USA). Murine TNF- $\alpha$  ( $5 \times 10^7$  U/mg) was obtained from Genzyme Corp. (Cambridge, Massachusetts, USA).

**Cell culture.** The murine T-cell line SAKRTLS12 was a gift from P. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA) and J. Lesley (The Salk Institute, La Jolla, California, USA). The human EBV-transformed B-lymphoblastoid cell line KCA (26) was kindly provided by L. Picker of the University of Texas Southwestern Medical Center (UTSWMC). Cells were maintained in DMEM and RPMI-1640, respectively, with high glucose, 10% FCS, 1 mM pyruvate, and 2 mM glutamine. Human T cells from peripheral blood (HuPBT) of healthy volunteers were obtained by passing mononuclear cells enriched by Ficoll-Hypaque gradient centrifugation over T-cell enrichment columns, according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, Minnesota, USA). Where indicated, cell lines and HuPBT were activated with 1 ng/mL PMA plus 500 ng/mL ionomycin for 18–24 hours at 37°C.

Lymph node cells were obtained from BALB/c mice approximately 8 weeks old. Five milliliters of freshly isolated lymph node cells ( $2 \times 10^6$  cells per mL) were

incubated at 37°C with immobilized hamster anti-CD3 (PharMingen, San Diego, California, USA) at 5  $\mu$ g/mL for 48 hours.

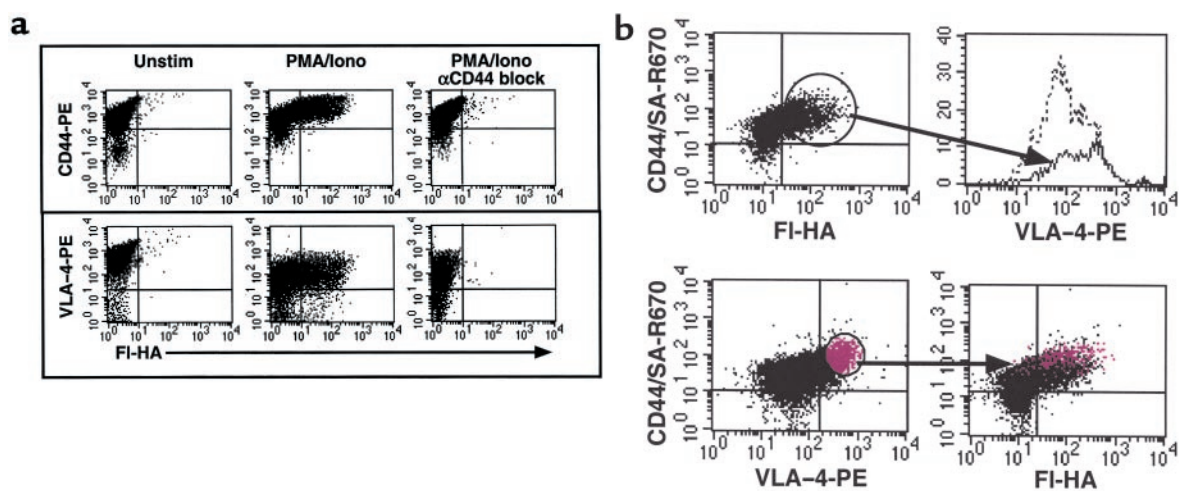
SVEC4-10 is an SV40-transformed murine lymph node endothelial cell line derived from lymph node stroma. Endothelial cells were maintained in a high-glucose solution of RPMI-1640, 15% FCS, 1 mM pyruvate, 2 mM glutamine, and 50  $\mu$ M  $\beta$ -mercaptoethanol. For rolling assays, cells were plated at 30% confluence and then incubated for 48–72 hours on 30-mm tissue culture dishes. TNF- $\alpha$  (10 ng/mL) was included in the final 18 hours of incubation to optimize levels of surface HA expression (27).

**Antibodies and soluble proteins.** Hybridomas producing HA-blocking rat anti-mouse CD44 (clone KM81) and blocking rat anti-mouse LFA-1 (clone M17/4) were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Blocking rat anti-mouse ICAM-1 (clone YN1/1.7) was provided by P. Thorpe (UTSWMC). Antibodies were purified from tissue culture supernatants by protein A-Sepharose column chromatography. Rat anti-mouse VLA-4 (anti- $\alpha 4$ ; clone PS/2) was kindly supplied by P. Kincade. Phycoerythrin (PE) and biotin conjugates of rat anti-mouse CD44 (non-HA blocking clone IM7) were purchased from PharMingen for FACS<sup>®</sup> analysis.

HA-blocking mouse anti-human CD44 (clone 515) was kindly provided by G. Kansas (Northwestern University Medical School, Chicago, Illinois, USA) (28, 29). Blocking mouse anti-human VLA-4 $\alpha$  chain (clone L25) (30) was purified from mouse ascites. Blocking mouse anti-human LFA-1 (clone G43-25B) was purchased from PharMingen. The hybridoma producing blocking mouse anti-human ICAM-1 (clone R6.5.D6.E9.B2) was obtained from the American Type Culture Collection; antibody was purified from culture supernatants as above.

Streptavidin-RED670 (SA-R670) was purchased from GIBCO BRL (Gaithersburg, Maryland, USA). PE-conjugated goat anti-rat immunoglobulin (GaRlg-PE) and goat anti-mouse immunoglobulin (GaMlg-PE) were obtained from Caltag Laboratories Inc. (Burlingame, California, USA).

Recombinant soluble human VCAM-1 and ICAM-1 (sVCAM-1 and sICAM-1) were purchased from R&D Systems Inc. A cell line producing recombinant murine sICAM-1 was a gift from F. Takei (University of British Columbia, Vancouver, Canada). Mouse sICAM-1 was purified at 4°C by passing culture supernatant through an anti-ICAM-1 antibody affinity column. The column was extensively washed with 0.15 M NaCl and 10 mM Tris at pH 7.5; bound sICAM-1 was eluted with 0.15 M NaCl and 50 mM diethylamine at pH 11.0. The eluate was immediately neutralized with a 0.1 $\times$  volume of 1 M Tris at pH 6.0. Eluted material was concentrated and exchanged into PBS using Amicon Centricon 30 microconcentrators (Millipore Corp., Beverly, Massachusetts, USA). The purity of sICAM-1 was confirmed by SDS-PAGE, and the concentration was determined using a DC Protein Assay Kit (Bio-Rad Labora-



**Figure 1**

FI-HA binding by HUPBT. (a) Untreated cells (Unstim) or cells treated with PMA and ionomycin for 18 hours were stained with FI-HA plus either CD44-PE or VLA-4/GaRig-PE as indicated, and then analyzed by 2-color FACS<sup>®</sup>. Few FI-HA<sup>+</sup> cells are seen among untreated HUPBT cells, whereas a distinct population of positive cells is seen after activation. FI-HA-binding cells are found predominantly in both the CD44<sup>+</sup> (top) and VLA-4<sup>+</sup> (bottom) populations. FI-HA binding by both CD44<sup>+</sup> and VLA-4<sup>+</sup> activated T cells is blocked by incubation with blocking anti-CD44 before staining. (b) Three-color immunofluorescence analysis of activated T cells demonstrates that the CD44<sup>hi</sup>/FI-HA<sup>+</sup> population is also the VLA-4<sup>hi</sup> population. Top: Histograms show the VLA-4-PE staining of cells stained with FI-HA, VLA-4-PE, and CD44/SA-R670. The VLA-4-PE staining of gated CD44/FI-HA double-positive cells (circled) is indicated by the solid histogram; the dashed histogram reflects the VLA-4-PE staining of the total population. Bottom: The CD44/VLA-4 double-positive population (red, circled) is contained predominantly in the CD44/FI-HA double-positive population.

tories Inc., Hercules, California, USA). Integrity of the purified sICAM-1 was verified by inhibition of anti-ICAM-1 staining of ICAM-1<sup>+</sup> cells and by LFA-1-dependent adhesion of activated murine T cells on sICAM-1-coated dishes in a static adhesion assay as described (10, 27) (data not shown).

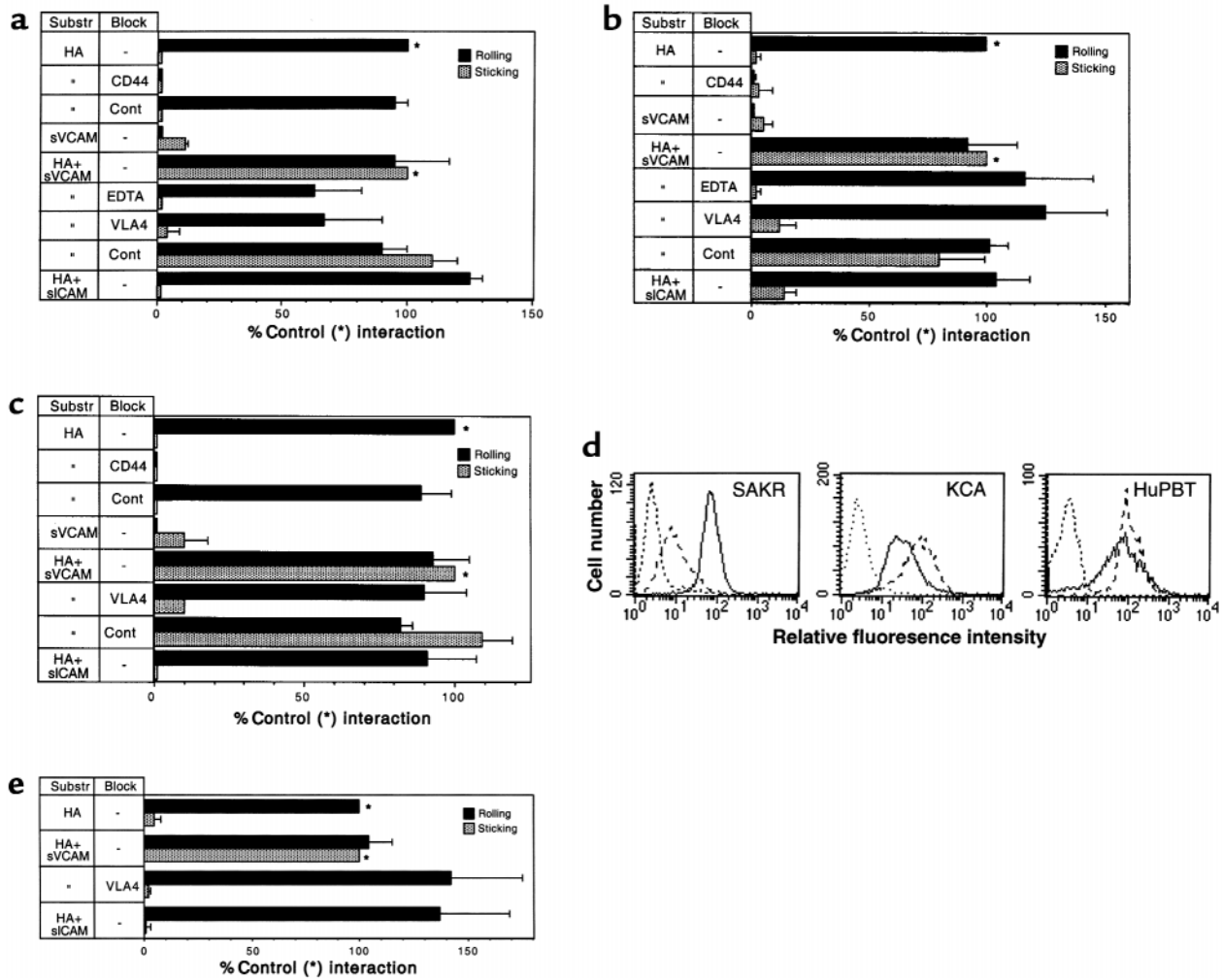
**FACS<sup>®</sup> analysis.** Unstimulated or PMA and ionomycin-activated lymphoid cells ( $5 \times 10^5$ ) were stained with antibody for 10 minutes on ice and then washed with 1 mL of PBS and 5% FCS. When indicated, cells were incubated with secondary antibody or SA-R670 for an additional 10 minutes and washed again. For blocking studies, a saturating amount of unlabeled monoclonal antibody 515 (which does not cross-block the staining of IM7) was preincubated with cells before addition of fluoresceinated-HA (FI-HA). Data were collected on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) and were analyzed using CellQUEST 3.1 software (Becton Dickinson Immunocytometry Systems).

**Adhesion assay under flow conditions.** Rolling and firm adhesion of lymphocytes was assessed on a parallel plate flow chamber under physiologic flow conditions as described (10). Briefly, a 35-mm tissue culture dish (Corning Glass Works, Corning, New York, USA) was coated with 1 mL of 2.5 mg/mL HA in PBS overnight at 4°C or with 1 mL sVCAM-1 (0.5–1.0 μg/mL) or sICAM-1 (1 μg/mL) in a solution of 10 mM NaHCO<sub>3</sub> in PBS (pH 9.1) for 2 hours at room temperature on a rocking platform. Plates coated with HA plus sVCAM-1 or HA plus sICAM-1 were first incubated overnight

with HA followed by a 2-hour incubation with sVCAM-1 or sICAM-1. Plates were blocked with 1 mL FCS for 1 hour at 4°C before use, and then rinsed 3 times with PBS. No tethering, rolling, or firm interactions were seen on plates coated with VCAM-1 or ICAM-1 alone at these concentrations, consistent with previous reports perfusing T cells at physiologic shear forces (1.8 dynes/cm<sup>2</sup>) over VCAM-1-coated plates at concentrations up to 15 μg/mL (31). Direct firm adhesion in this system was only detectable on plates coated with sVCAM-1 alone at concentrations above 3 μg/mL. Monolayers of SVEC4-10 endothelial cells were used at confluence. Experiments were carried out at a wall shear stress of 2.0 dynes/cm<sup>2</sup> for mouse cells or 1.7 dynes/cm<sup>2</sup> for human cells. Blocking studies were carried out by incubating the cells with saturating concentrations of antibody for 10 minutes before the rolling assay.

For quantitation, rolling cells from at least 4 separate fields were counted for 1–2 minutes per field. Firm adhesion (sticking) was determined by counting total firmly adherent cells on 4 fields after 4 minutes of flow. At least 3 samples were analyzed for each interaction condition. The average was calculated and results are shown as percentage of positive control interaction (rolling or sticking). For experiments on endothelial cell monolayers, the data are expressed as cells/field per minute (rolling) and bound cells/field (sticking).

**Short-term homing of lymphocytes in vivo.** BALB/c mice were raised in the animal colony at UTSWMC. All in vivo experimental groups consisted of 3 or more mice. Donor



**Figure 2**

Primary (rolling) and secondary (firm) adhesion of murine SAKR (a), human KCA (b), and HuPBT (c) cells on soluble substrates under physiologic shear stress. Cells were applied to feed solution already equilibrated under flow and then perfused over the indicated individual or combined substrates in the parallel plate flow assay. Rolling and sticking were analyzed as described in Methods, and the number of rolling cells/field per minute and the number of bound cells/field were determined for each substrate. Results are compared to control interactions (indicated with asterisks), and presented as a percentage of the total (100%) control. Mean absolute values (100%) for rolling (given in cells/field per minute) and sticking (given in cells/field), respectively, were: SAKR, 14.6 and 9.7; KCA, 40 and 35.5; HuPBT, 9.6 and 9.8. For blocking, anti-CD44, anti-VLA-4, anti-LFA-1 (Cont), or EDTA was added to the cell suspension before introduction into the flow system. With each cell type, firm adhesion occurred only on plates coated with HA plus sVCAM-1. All interactions were blocked with anti-CD44, whereas only anti-VLA-4 was able to block sticking without affecting rolling. EDTA also inhibited firm adhesion. (d) Expression of integrins VLA-4 and LFA-1 on SAKR, KCA, and HuPBT cells. Cells were stained with primary antibody plus GaRIg-PE or GaMIg-PE for FACS<sup>®</sup> analysis. All 3 cell types were positive for both VLA-4 (dashed lines) and LFA-1 (solid lines). Stippled lines represent control staining. (e) Primary and secondary adhesion of anti-CD3-activated murine lymph node cells on soluble substrates under physiologic shear stress. Assays were performed and analyzed as above. Mean absolute values (100%) for rolling and sticking, respectively, were 15.1 cells/field per minute and 72.8 cells/field. Substr, substrate; Block, blocking antibody or EDTA.

and recipient mice, 8–12 weeks old, were injected with 50  $\mu$ g SEB in 500  $\mu$ L PBS or with PBS alone. Lymphocytes from mesenteric lymph nodes of donor mice were isolated, labeled with CFDA for 20 minutes at room temperature, and washed with PBS. Twenty hours after SEB injection of recipient mice,  $10^7$  fluorescent donor cells in 500  $\mu$ L HBSS were injected into the tail vein of each recipient mouse. For blocking experiments, antibody was added at saturating concentration (100  $\mu$ g) to the cell suspension before injection. Ninety minutes after

intravenous cell injection, recipient mice were sacrificed. All cells in the peritoneal cavity were collected by lavage with 5 mL of RPMI-1640 at 37°C, and were analyzed by FACS<sup>®</sup> for the presence of fluorescent donor cells. At least 10 animals were analyzed for each condition.

## Results

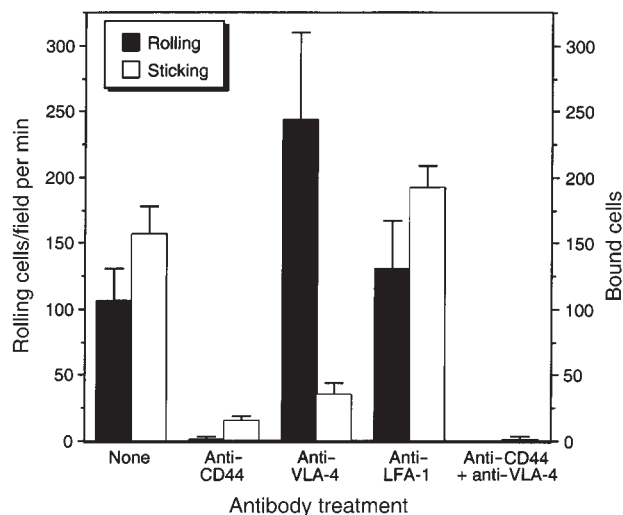
*The activated form of CD44 is associated with elevated VLA-4 expression on activated leukocytes.* The 3 cell types used for these studies — the murine T cell line SAKRTLS12, the

human lymphoblastoid line KCA, and normal HuPBT – were chosen based on their coexpression of the common leukocyte integrins  $\alpha 4\beta 1$  (VLA-4) and  $\alpha L\beta 2$  (LFA-1), as well as for their ability to convert CD44 to its activated (HA-binding) form after polyclonal stimulation (10, 32). Although CD44 is expressed at significant levels on resting HuPBT, it does not bind FI-HA (Figure 1a). After overnight polyclonal stimulation, a significant percentage of the CD44<sup>+</sup> cell population acquires the ability to bind FI-HA (10, 32). As shown, the number of cells with the FI-HA-binding phenotype was particularly increased among the VLA-4<sup>+</sup> cells after stimulation, although overall VLA-4 levels diminished somewhat (Figure 1a). Thus, activation of CD44 to the HA-binding form is preferentially associated with cells expressing higher levels of VLA-4. This is further detailed in Figure 1b, where 3-color analysis demonstrates that CD44<sup>+</sup> FI-HA-binding cells express VLA-4 at higher levels than are seen in the total population (top). Likewise, it can be seen that CD44<sup>hi</sup>/VLA-4<sup>hi</sup> double-positive cells are predominantly positive for FI-HA binding (bottom), a finding also seen in SEB-stimulated mouse lymphocytes (see Figure 4a).

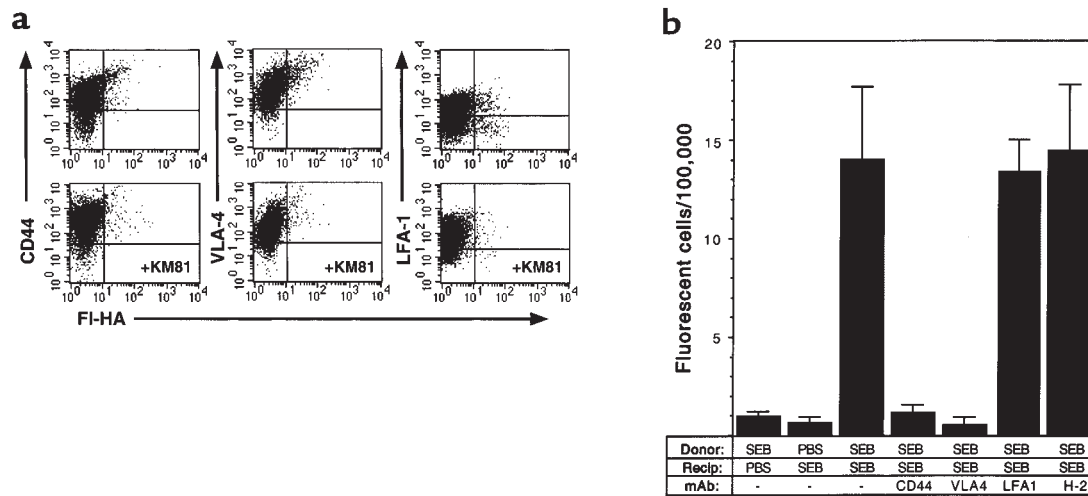
*CD44/HA-mediated primary adhesion is followed by VLA-4/VCAM-1-dependent secondary adhesion under shear forces.* To examine firm adhesion directly under laminar flow after CD44-initiated rolling adhesion, plates were coated with HA simultaneously with the VLA-4 and LFA-1 integrin ligands sVCAM-1 and sICAM-1, respectively. Thus, activated murine and human lines and normal human T cells were analyzed for both primary and secondary adhesion under laminar flow on the substrates HA, sVCAM-1, HA plus sVCAM-1, and HA plus sICAM-1. Results of these analyses are shown in Figure 2. Activated SAKR cells exhibit primary adhesion on plates coated with HA alone, and this rolling can be completely inhibited with blocking anti-CD44 antibody, but not with isotype-matched control anti-LFA-1 (Figure 2a) (10). Both of the human cell populations (KCA and HuPBT) also rolled on HA after stimulation with PMA and ionomycin; this was also CD44 dependent (Figure 2, b and c). No substantial firm adhesion was noted with any of the cells tested on HA substrate alone, again confirming that the CD44/HA interaction is involved primarily in rolling adhesions.

On sVCAM-1 alone, detectable but minimal direct firm adhesion, and essentially no rolling, was observed (Figure 2). In contrast, when each of these cell populations was assayed on a substrate of HA plus sVCAM-1, significant numbers of firmly adherent cells were seen in addition to rolling cells (Figure 2, a–c), indicating that sVCAM-1 can function as a secondary adhesion ligand after primary adhesion mediated by CD44/HA. In addition, 22–46% of total adherent cells at 4 minutes were observed to convert from rolling to firmly adherent. To confirm that the lymphocyte receptor for this interaction was the integrin VLA-4, cells were pretreated with anti-VLA-4 before the shear stress assay. Anti-VLA-4 antibody treatment (M17/4 for mouse

cells and L25 for human cells) completely inhibited firm adhesion in each case, but had little effect on CD44-dependent rolling. Control antibody also had no effect on either rolling or sticking. Also consistent with an integrin-mediated interaction (which requires the presence of divalent cations) is the fact that firm adhesion was preferentially inhibited by inclusion of 5 mM EDTA in the assay buffer (Figure 2, a and b). Moreover, the VLA-4 dependence of firm adhesion was not due to differences between expression levels of VLA-4 and LFA-1, because each cell type expressed comparable levels of both integrins, with SAKR cells expressing significantly more LFA-1 than VLA-4 (Figure 2d). In contrast, no firm adhesion was seen on plates coated with both HA and sICAM-1. Neither direct firm adhesion of cells to sICAM-1 alone nor firm adhesion after CD44-mediated primary adhesion on plates coated with both HA and sICAM-1 was observed, even at 10-fold higher sICAM-1 concentrations (data not shown). Thus, although expressed on all 3 cell types, LFA-1 does not appear to play a significant role in mediating firm adhesion under shear stress in the CD44-initiated pathway. However, it should be noted that LFA-1 on these populations was competent to bind ICAM-1 on coated plates in both static and nonstatic (rotational, low shear) adhesion assays (27, 33) (data not shown), consistent with prior reports (34–38). It is also of note that for these activated lymphocyte populations,



**Figure 3** Primary and secondary adhesion of SAKR cells on SVEC4-10 endothelial cells under physiologic shear stress. SAKR cells were applied to feed solution already equilibrated under flow at a wall shear stress of 2.0 dynes/cm<sup>2</sup> and the solution was perfused over the monolayer in the parallel plate flow assay. Rolling and sticking were analyzed as described, and the number of cells/field per minute rolling and number of bound cells/field were determined. For blocking, anti-CD44, anti-VLA-4, or anti-CD44 together with anti-VLA-4 was added to the cell suspension before introduction into the flow system. Rolling and firm adhesion were both blocked by anti-CD44, whereas only anti-VLA-4 was able to block firm adhesion. Data shown are from a representative of 3 experiments.



**Figure 4**

VLA-4 participates in the short-term homing of activated lymphocytes to an inflamed site. (a) Mesenteric lymph node cells from SEB-treated mice were stained with FI-HA plus either CD44-PE, VLA-4/GaRlg-PE, or LFA-1/GaRlg-PE as indicated. FI-HA-binding cells are present predominantly in both the CD44<sup>+</sup> and VLA-4<sup>+</sup> populations, but are distributed between both LFA-1<sup>+</sup> and LFA-1<sup>-</sup> populations. FI-HA binding is blocked by incubation with blocking anti-CD44 before staining. (b) CFDA-labeled mesenteric lymph node cells from SEB- or PBS-treated mice were injected intravenously into recipient mice that had received an intraperitoneal injection of SEB 20 hours earlier. Cells from donor mice were injected without antibody or with HA-blocking anti-CD44 monoclonal antibody, blocking anti-VLA-4 $\alpha$ , blocking anti-LFA-1, or isotype-matched control anti-H-2 monoclonal antibody. Two hours after donor cell injection, peritoneal cavities of recipient mice were lavaged, and recovered cells were analyzed by FACS<sup>®</sup> for CFDA fluorescence. Data are shown as the number of fluorescent cells per 100,000 cells analyzed, and represent the mean  $\pm$  SEM of 3 separate experiments ( $\geq 3$  mice/group per experiment). No peritoneal accumulation of fluorescent cells was seen when either anti-CD44 or anti-VLA-4 antibody was added to the injected cell suspension. Anti-LFA-1 and control anti-H-2 had no effect on cell migration into peritoneal cavities.

rolling mediated by VLA-4/VCAM-1 was only minimally observed, although this capability has been demonstrated in previous studies (31, 39, 40). However, our observations are consistent with prior reports that rolling was not observed on isolated VCAM-1 substrate using activated lymphoid cells. Immediate firm adhesion was seen, but only at lower shear forces (0.3–0.7 dynes/cm<sup>2</sup>) (31). Direct firm adhesion was not observed on VCAM-1 under the conditions used in these studies. Therefore, using soluble substrates, primary adhesion mediated by activated CD44/HA appears to be followed by secondary adhesion mediated by VLA-4/VCAM-1 under physiologic shear stresses; this sequence is maintained in both mouse and human.

To determine whether a more physiologic stimulus resulted in a similar adhesion pathway, murine lymph node cells were stimulated directly through the T-cell receptor with anti-CD3. This alternative stimulation resulted in a pattern that was virtually identical to that seen with PMA and ionomycin. Stable arrest was overwhelmingly dependent on VLA-4 after CD44-initiated rolling adhesion (Figure 2e). Thus, a more physiologic protocol confirms the pattern found with the above populations.

*VLA-4/VCAM-1-mediated firm adhesion follows CD44/HA primary adhesion on endothelial monolayers.* We have previously demonstrated CD44-mediated rolling of T-cell populations on endothelial cell lines and primary endothelial cultures expressing HA. Moreover,

endothelial HA levels are induced by proinflammatory stimuli; this results in increased CD44/HA-mediated adhesive interactions under conditions simulating physiologic shear stress (10, 27, 41). However, only primary adhesion was addressed in those experiments. Therefore, we sought to confirm the observations on isolated substrate, and to further examine the role of VLA-4-mediated secondary adhesion after CD44-mediated primary adhesion on endothelial monolayers expressing VCAM-1. To this end, we tested activated SAKR cells under laminar flow on monolayers of TNF- $\alpha$ -treated murine SVEC4-10 endothelial cells, which express both VCAM-1 and ICAM-1 (41). As shown in Figure 3, both primary and firm adhesion of SAKR cells occurred on this monolayer. Essentially all rolling interactions were blocked by anti-CD44, and very little firm adhesion was evident when CD44-mediated rolling was blocked, indicating a minimal role for VLA-4 in primary adhesion on these endothelial cells. Furthermore, very little firm adhesion was observed when CD44 interactions were blocked, indicating a minimal role of VLA-4/VCAM-1 in direct firm adhesion in this system. In contrast, firm adhesion was blocked more than 80% by anti-VLA-4, with compensatory increases in rolling interactions, whereas the control anti-LFA-1 (also expressed on SAKR cells, Figure 2d) had no significant effect on either sticking or rolling. A combination of anti-CD44 and anti-VLA-4 removed almost all interactions. Thus, the adhesion interactions observed

using soluble substrates can be reproduced under more physiologic circumstances using endothelial cells expressing the appropriate ligands, HA and VCAM-1.

*VLA-4 participates in homing of superantigen-activated lymphocytes to an inflamed site in vivo.* We showed previously that activated CD44 interactions with HA are critical to the traffic of superantigen-activated T cells from the bloodstream into inflamed peritoneal cavities in a short-term homing assay (13, 41). We conducted similar studies to determine if VLA-4 on lymphocytes also participates in this in vivo trafficking model. Draining mesenteric lymph node cells, from mice treated with SEB 20–24 hours before sacrifice, were used as donor cells. FACS<sup>®</sup> analysis of these cells demonstrated a subpopulation bearing activated CD44, and a similar population expressing high levels of VLA-4 that preferentially bound Fl-HA (Figure 4a). Furthermore, as with activated normal human T cells (Figure 1), the HA-binding cells were concentrated in the population coexpressing high levels of both CD44 and VLA-4 (data not shown). In contrast, HA-binding cells were more evenly distributed across the expression levels of LFA-1, similar to results seen with activated human T cells (data not shown).

To assess homing capabilities, CFDA-labeled activated donor lymph node cells were injected intravenously into recipient mice treated intraperitoneally with SEB to create an inflamed peritoneal site. Migration of fluorescent donor cells from the blood into the inflamed site was assessed by FACS<sup>®</sup> analysis of cells recovered by lavage of the peritoneal cavity 2 hours after intravenous injection. As shown in Figure 4b, significant numbers of fluorescent donor cells were found in recipient peritoneal cavities compared with either unactivated donor cells or activated cells injected into untreated recipients; these cells have previously been shown to be the activated V $\beta$ 8-bearing T-cell subset (12, 41). Fluorescent donor-cell migration into recipient peritoneal cavities is almost completely prevented by coadministration of either blocking anti-CD44 antibody or blocking anti-VLA-4 antibody (Figure 4b). However, neither anti-LFA-1 nor control anti-H-2 antibodies had any effect on cell migration. Thus, in an in vivo homing model that depends on CD44/HA interactions, VLA-4 also appears to be essential in the trafficking of activated lymphocytes into an inflamed peritoneal site, consistent with a model in which CD-44 and VLA-4 act sequentially.

## Discussion

VLA-4 is expressed on T cells, monocytes, and eosinophils (and according to some studies, on neutrophils), and has been shown to have a role in many inflammatory models involving all of these cell types (42–48). The VLA-4/VCAM-1 interaction has been shown to be important for lymphocyte/endothelial interactions at several inflammatory sites. Examples include entry of CD4<sup>+</sup> T cells into the brain in experimental allergic encephalomyelitis (42, 49), in rheumatoid synovium (50, 51), and for the development of autoimmune diabetes (52). Our prior evidence in an

antigen-specific contact hypersensitivity response suggested that this integrin operates at the level of effector T cells, which are in turn necessary for the recruitment of nonspecific effector cells (53). This is consistent with other contact hypersensitivity studies in mice and primates (54–56), while in a rat model of skin delayed-type hypersensitivity, both anti-VLA-4 and LFA-1 were required for maximal in vivo blocking (57). The studies presented here suggest that the lymphocyte-trafficking roles attributed to VLA-4 could in some situations follow CD44 tethering and rolling on endothelial HA.

Integrins containing the  $\alpha$ 4 chain have been demonstrated to be quite versatile with respect to the adhesion cascade. In addition to firm arrest (4), these integrins are able to mediate tethering and rolling. In contrast, we observed no measurable rolling, although, as noted, we did detect some direct attachment on VCAM-1 alone (Figure 2), consistent with earlier observations (31). In addition,  $\alpha$ 4 $\beta$ 7 could mediate firm arrest but not rolling of preactivated lymph node cells in Peyer's patch high endothelial venules in vivo (58). Analogous to the CD44-initiated pathway described here, VLA-4 has been reported to mediate firm adhesion of CD4<sup>+</sup> T cells after P-selectin-driven primary adhesion (59). Moreover, in some in vivo models, rolling mediated by  $\alpha$ 4 and integrins/VCAM-1 has been shown to have a detectable but very small role compared with all selectin interactions (60). Thus, the CD44/HA primary adhesion interaction may be preferentially used in conjunction with VLA-4 by activated cell populations, whereas resting cells on which CD44 is not activated may use  $\alpha$ 4 integrins for both adhesion steps during extravasation, or may act in conjunction with selectins to effect firm adhesion.

The predilection for the use of VLA-4 but not LFA-1 in these experiments is striking. Very little contribution by LFA-1 under laminar flow (Figures 2 and 3) or for in vivo homing (Figure 4) could be detected, even though it was clear that LFA-1 was active after cell stimulation in static binding assays to purified mouse or human sICAM-1 (data not shown). This latter point is in agreement with previous reports showing active LFA-1 after cell stimulation (34–36, 61, 62). In addition, in laminar flow analysis applying half the shear used in other experiments, direct firm adhesion could be detected on ICAM-1 alone (data not shown). This further suggests that LFA-1 on recently activated cells could potentially participate in secondary adhesion after primary adhesion is initiated. LFA-1 is essential for the homing of naive lymphocytes into peripheral lymph nodes after L-selectin-mediated tethering and rolling (63). Thus, LFA-1 may generally be used for recruitment of naive rather than activated T cells. The mechanism for this selectivity requires further investigation; however, cross-talk between integrins has been reported. For example, interaction of LFA-1 selectively reduces adhesion mediated by  $\alpha$ 4 $\beta$ 1, allowing coordination of integrin function on T cells (37), although our evidence suggests the reverse: that  $\alpha$ 4 $\beta$ 1 may suppress LFA-1 in recently activated lymphoid cells.



In summary, these studies were undertaken to examine the mechanism by which CD44/HA-mediated tethering and rolling converts to firm adhesion. Both mouse and human activated T-cell populations demonstrate a dominant role for VLA-4 in this step under conditions of laminar flow, and superantigen-activated T cells show dependence on both VLA-4 and CD44 in an in vivo mouse model of peritoneal inflammation. LFA-1 does not appear to participate substantially in this pathway in these systems. These results help to clarify the molecular basis for extravasation of activated T cells, which are critical to the initiation and perpetuation of autoimmune and chronic inflammatory states. They may further explain the frequent association of both CD44 and VLA-4 interactions with chronic inflammatory states and suggest that therapeutic strategies targeting both of these receptors may be beneficial.

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