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Leukocyte Rolling Velocities and Migration Are Optimized by Cooperative L-Selectin and Intercellular Adhesion Molecule-1 Functions

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Selectin family members largely mediate initial tethering and rolling of leukocytes on vascular endothelium, whereas integrin and Ig family members are essential for leukocyte firm adhesion. To quantify functional synergy between L-selectin and Ig family members during leukocyte rolling, the EA.hy926 human vascular endothelial line was transfected with either fucosyltransferase VII (926-FI/VII) cDNA to generate L-selectin ligands alone or together with ICAM-1 cDNA (926-FI/VII/ICAM-1). The ability of transfected 926 cells to support leukocyte interactions was assessed in vitro using parallel plate flow chamber assays. Lymphocyte rolling on 926-FI/VII cells was increased by ~70% when ICAM-1 was expressed at physiological levels. Although initial tether formation was similar for both cell types, lymphocyte rolling was 26% slower on 926-FI/VII/ICAM-1 cells. Pretreatment of lymphocytes with an anti-CD18 mAb eliminated the increase in rolling, and all rolling was blocked by anti-L-selectin mAb. In addition, rolling velocities of lymphocytes from CD18-hypomorphic mice were 48% faster on 926-FI/VII/ICAM-1 cells, with a similar reduction in rolling frequency relative to wild-type lymphocytes. CD18-hypomorphic lymphocytes also showed an ~40% decrease in migration to peripheral and mesenteric lymph nodes during in vivo migration assays compared with wild-type lymphocytes. Likewise, wild-type lymphocyte migration to peripheral lymph nodes was reduced by ~50% in ICAM-1−/− recipient mice. Similar to human lymphocytes, human neutrophils showed enhanced rolling interactions on 926-FI/VII/ICAM-1 cells, but also firmly adhered. Thus, in addition to mediating leukocyte firm adhesion, CD18 integrin/ICAM-1 interactions regulate leukocyte rolling velocities and thereby optimize L-selectin-mediated leukocyte rolling. The Journal of Immunology, 2002, 169: 4542-4550.

Lymphocyte migration and leukocyte recruitment into sites of inflammation require leukocyte interactions with vascular endothelium under conditions of shear flow. L-selectin (CD62L), constitutively expressed by most leukocytes, and P- and E-selectin, expressed by activated endothelial cells, predominantly mediate initial leukocyte tethering and rolling along the endothelium (1). L-selectin ligands are constitutively expressed by peripheral lymphoid tissues and are induced on vascular endothelium by inflammatory cytokines (2–4). The original paradigm of leukocyte adhesion dictated that rolling and firm adhesion were separate and sequential steps (5). L-selectins express $\beta_2$ integrins, including LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), which interact with endothelial ligands, including ICAM-1 (CD54) (6, 7). ICAM-1 is constitutively expressed at low levels on resting endothelial cells, but is rapidly up-regulated during inflammation, resulting in increased leukocyte/endothelial cell adhesion (8). Adhesion molecules including $\alpha_\beta_1$ (CD49d/CD29) and $\alpha_\beta_2$ integrins as well as hyaluronan receptors (CD44) can support leukocyte rolling under in vitro conditions of low shear stress (9–13). In addition, LFA-1 expressed in erythroleukemia cells can mediate rolling in vitro on immobilized ICAM-1 under shear flow, but fails to support firm adhesion (14). However, T lymphocytes and neutrophils do not interact with immobilized ICAM-1 under physiological shear flow without prior cellular activation (15), and leukocytes are unable to roll in vivo independent of selectins except under reduced shear (16). Thus, the selectins are thought to primarily mediate leukocyte capture and rolling, while integrins can mediate rolling in vitro on plastic-immobilized ligands under specialized conditions.

In vivo, leukocyte rolling on vascular endothelium results from a complex series of overlapping adhesion and signaling events mediated by vascular adhesion molecules and chemokines. As an example, the selectins, ICAM-1 and CD18 integrins appear to have overlapping functions during optimal selectin-mediated rolling in vivo (17–21) and during leukocyte migration to sites of inflammation (18). Leukocyte rolling mediated by P-selectin does not require L-selectin or ICAM-1 expression, but ICAM-1 expression is required for P-selectin to mediate rolling at characteristic velocities in vivo (17, 20). By contrast, ICAM-1 expression is essential for leukocyte rolling mediated by L-selectin in the absence of P-selectin expression (20). Thus, L-selectin can only mediate leukocyte rolling in vivo when ICAM-1, P-selectin, or both are expressed, while P-selectin can mediate leukocyte rolling in the absence of L-selectin and ICAM-1 expression, albeit at significantly faster velocities. As a result, leukocyte rolling velocities are significantly increased in ICAM-1-deficient (ICAM-1−/−) mice during inflammation (17–21), and both LFA-1 and Mac-1 contribute to CD18-dependent rolling velocities (22). Although ICAM-1 is necessary for neutrophil adhesion to unstimulated endothelium, it has also been suggested that ICAM-1 is not significantly involved in either slow rolling (23) or chemoattractant-induced firm adhesion of leukocytes in inflamed venules (24). These studies indicate the complex and overlapping functions of adhesion molecules during in vivo inflammation that have collectively led to the
the concept that CD18 integrin and ICAM-1 expression support optimal P- and L-selectin-mediated leukocyte rolling to facilitate firm adhesion and transmigration.

It remains difficult to reconcile the in vivo data with the in vitro studies examining integrin-mediated rolling under low shear stress in part because the vast majority of intravital microscopy studies analyze only neutrophil recruitment. It is also difficult to distinguish between lymphocyte, or lymphocyte subset, and neutrophil rolling in vivo. Moreover, multiple adhesion molecules and chemokines regulate leukocyte recruitment and rolling in vivo. For these reasons, it has been difficult to quantify the extent that the integrins contribute to leukocyte tethering and subsequent rolling on vascular endothelial cells under defined conditions. Therefore, an in vitro flow chamber assay using vascular endothelial cells was established in the current study to directly quantify the functional synergy between L-selectin and Ig family members during lymphocyte and neutrophil rolling. The EA.hy926 human endothelial cell line (926 cells)\(^1\) (25) was transfected with either fucosyltransferase VII (926-FtVII) alone or together with ICAM-1 (926-FtVII/ICAM-1) and tested for its ability to support leukocyte rolling and adhesion. One advantage of this system is that the effects of other adhesion molecules, including P-selectin, E-selectin, and \(\alpha\)L integrins, can be dismissed, since leukocyte rolling on 926-FtVII cells was completely L-selectin dependent (26). The results confirm that L-selectin alone initiates initial leukocyte/endothelial cell interactions, but show that ICAM-1 expression facilitates L-selectin-mediated rolling of lymphocytes and neutrophils under physiological shear flow.

Materials and Methods

Mice

C57BL/6 (B6), ICAM-1\(^{-/-}\) (27), and CD18-hypomorphic (CD18\(^{hypo}\)) (28) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). L-selectin-deficient (L-selectin\(^{-/-}\)) and L-selectin-heterozygous (L-selectin\(^{+/-}\)) mice backcrossed onto the B6 background for \(\geq 10\) generations were previously described (29). Mice were housed in specific pathogen-free barrier facilities. All procedures were approved by the animal care and use committee of Duke University Medical Center.

Antibodies

The anti-human ICAM-1 mAb HAE-4b (2) was used as diluted ascites fluid. The HECA-452 hybridoma was obtained from American Type Culture Collection (Manassas, VA) and was used as culture supernatant. The anti-human CD18 mAb (10F12) (30); LAM1–3 anti-human L-selectin mAb (31); LAM1–101, LAM1–116, and LAM1–118 anti-L-selectin mAbs (32); the anti-human ICAM-2 mAb (CBR-IC2/1) (33); and the HB15c (anti-CD83) mAb that was used as an isotype control mAb (34) were used as purified Abs. FITC-conjugated goat anti-rat IgM and anti-mouse IgG1 Abs and anti-mouse CD25 mAb were obtained from Southern Biotechnol- ogy Associates (Birmingham, AL). Immunofluorescence was analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Generation of 926 cell lines

926 cells were a gift from Dr. C.-J. Edgell (University of North Carolina, Chapel Hill, NC). 926-FVII cells were generated by transfecting 926 cells with \(\alpha\L,3\)-fucosyltransferase (FrVII) cDNA (from Dr. B. Weston, University of North Carolina) as previously described (26). 926-ICAM-1 cells were generated by transfecting 926 cells with ICAM-1 cDNA (from Dr. D. Staunton, Center for Blood Research, Boston, MA). 926-FVII/ICAM-1 cells were generated by cotransfection with FrVII and ICAM-1 cDNAs. To assess cell surface Ag expression, transfected cells were incubated with either HECA-452 or HAE-4b mAb for 20 min on ice, with mAb binding assessed using FITC-conjugated goat anti-rat Ig or goat anti-mouse IgG1 Ab, respectively. ICAM-1 and/or HECA-452 cells with similar mean fluorescence intensities were isolated by fluorescence-activated cell sorting and used for all experiments. In some experiments 926 cells were cultured in medium containing recombinant human TNF-\(\alpha\) (10 U/ml; Genzyme, Cambridge, MA) for 6 h before immunofluorescence staining.

Lymphocyte and neutrophil isolation

Heparinized blood from normal volunteers was isolated using protocols approved by the human use committee of Duke University. Lymphocytes were isolated by density gradient centrifugation using Lymphoprep (Ny- comed, Oslo, Norway) and represented >95% of the cells isolated. Blood neutrophils were isolated by density gradient centrifugation using Mono- poly resolving medium (ICN Biomedicals, Costa Mesa, CA). Mouse lymphocytes from peripheral lymph nodes (PLN; inguinal, axillary, and cervical) and mesenteric lymph nodes (MLN) were isolated from B6, CD18\(^{hypo}\), L-selectin\(^{-/-}\), and L-selectin\(^{+/-}\) mice. PLN and MLN lymphocytes were mixed at a 1:1 ratio and used for in vitro flow chamber assays, except for lymphocytes from L-selectin\(^{-/-}\) mice, where MLN lymphocytes were dominant due to the limited number of PLN lymphocytes. To assess cell surface-L-selectin expression levels, PLN lymphocytes from B6 and CD18\(^{hypo}\) mice were stained with FITC-conjugated LAM1–116 mAb for 20 min on ice and analyzed by flow cytometry.

Leukocyte rolling under defined flow conditions

Leukocyte/endothelial cell interactions under physiologic flow conditions were assessed using an in vitro flow chamber as previously described (35). 926 cell monolayers were grown to confluence on 25-mm circular glass coverslips and mounted in a parallel plate flow chamber. For most experiments flow medium was drawn through the chamber at a calculated shear stress of 1.85 dyne/cm\(^2\) with a syringe pump (Harvard Apparatus, Natick, MA). Leukocytes (10\(^7\) cells) were suspended in PBS containing 0.75 mM CaCl\(_2\), 0.75 mM MgCl\(_2\), and 0.5% (v/v) BSA and then perfused through the chamber for a 10-min period. Cell rolling was observed using an inverted phase contrast microscope (Olympus, Lake Success, NY) and was videotaped using a CCD video camera (Hitachi Denshi, Tokyo, Japan) with a SuperVHS video recorder (model SVO-9500 MD, Sony, New York, NY) and an attached time-date generator (Microimage Video Sales, Bechtlehs, CA). Rolling cells were field counted on a 400- \(\mu\)m-wide line scanned with \(0.16 \text{mm}^2\) on a video monitor were counted at 10 random time points after an 8-min perfusion period. For neutrophils, multiple random fields were recorded for at least 10 s at the end of the 10-min perfusion period. The total number of firmly adherent neutrophils within each 0.16-\(\text{mm}^2\) field was determined by analyzing the videotapes, with a minimum of 10 fields analyzed for each experiment. Stable adhesion was defined as attachment without movement for a minimum of 10 s. For measuring rolling and adherent leukocytes at different shear stresses, flow was initiated at 3.0 dyne/cm\(^2\), and shear stress was reduced as indicated at 1.5-min intervals by changing the flow rate through the flow chamber. The number of rolling leukocytes that crossed a 400- \(\mu\)m-wide field over a 10-s period was counted in randomly chosen fields at each shear stress. For neutrophils, the number of neutrophils that newly adhered during a 1-min period within a 0.16-\(\text{mm}^2\)-square field was also counted at each shear stress. For calculating velocities, the distance each cell traveled between two time points was measured and divided by the elapsed time. In experiments involving mAb treatments, leukocytes (10\(^7\) cells) were preincubated with each mAb (10 \(\mu\)g/ml) for 20 min at room temperature, diluted 10-fold, and perfused through the flow chamber.

In vivo migration assays

Migration assays were previously described (36). For single-color migration assays, single-cell suspensions were prepared from the spleens of wild-type mice. Erythrocytes were lysed in Tris-buffered 100 mM ammonium chloride solution. Splenocytes (5–10 \(\times\) 10\(^5\)) were incubated in 2 ml RPMI 1640 medium containing calcium-AM (0.125 \(\mu\)M for 1 h migration assays, 1 \(\mu\)M for 48 h migration assays; Molecular Probes, Eugene, OR), on ice for 30 min with gentle mixing every 5 min. Cells were then washed twice in 100 mM PBS, counted, and resuspended at 1 \(\times\) 10\(^5\) cells/ml in PBS. Lymphocytes (4 \(\times\) 10\(^7\) in 400 \(\mu\)l) were injected into the lateral tail vein of individual wild-type or ICAM\(^{-/-}\) recipient mice. One or 48 h after the injection, recipient mice were bled, and single-cell suspensions of lymphoid tissues were prepared. One thousand to 5000 calcein-labeled cells with the forward and side light scatter properties of mononuclear cells were stained by flow cytometry, and the total number of calcein-labeled cells from the tissue covered from individual lymphoid tissues was determined by multiplying the total cell counts for individual tissues by the frequency of calcein-labeled cells within the tissue.

\(^{1}\) Abbreviations used in this paper: 926, EA.hy926 cells; 926-FVII, 926 cells stably transfected with fucosyltransferase VII cDNA; 926-FVII/ICAM-1, 926 cells stably co-transfected with fucosyltransferase VII and ICAM-1 cDNAs; 926-ICAM-1, 926 cells stably transfected with ICAM-1 cDNA; CD18\(^{hypo}\), CD18-hypomorphic; MLN, mesenteric lymph node; PLN, peripheral lymph node.
For two-color migration assays, lymphocytes (either wild-type or CD18<sup>−/−</sup> splenocytes) were labeled with calcein-AM as described above. Internal control wild-type splenocytes were labeled with PKH26 (Sigma, St. Louis, MO). Briefly, splenocytes (5–10 × 10<sup>5</sup>) were resuspended in 1 ml PKH26 dye solution, and allowed to incubate at room temperature for 2 min. Labeling was stopped by the addition of 2 ml FCS. Cell suspensions were washed twice with PBS, counted, and then stored on ice, PKH26-labeled cells (2 × 10<sup>7</sup>) and calcein-labeled cells (2 × 10<sup>7</sup>) were mixed in a total volume of 400 μl for injection into the lateral tail vein of individual wild-type mice. An aliquot of the injected cell mixture was also analyzed by flow cytometry to calculate the injected ratio of calcein- to PKH26-labeled cells (R<sub>i</sub>). After 1 or 48 h, the mice were bled, single-cell suspensions of lymphoid tissues were prepared, and the percentages of calcein- and PKH26-labeled cells were determined by flow cytometric analysis. A minimum of 5000 PKH26-labeled cells were collected for each sample. The ratio of calcein-labeled test cells to PKH26-labeled internal control cells within tissues or blood (R<sub>i</sub>) was calculated and expressed as the ratio of R<sub>i</sub>/R<sub>f</sub> for each tissue.

Statistical analysis

Data are expressed as the mean ± SEM. Student’s t test was used to determine the significance of differences between sample means.

Results

ICAM-1 expression by 926 cells

Unactivated 926 cells do not express detectable levels of most vascular adhesion molecules including P-selectin, E-selectin, CD34, P-selectin glycoprotein ligand-1, vascular cell adhesion molecule, or vascular adhesion protein-1 (26, 37, 38). This makes 926 cells useful for measuring the contributions of individually expressed molecules to leukocyte adhesion. For example, 926-Ft-VII cells express sLex-related cell surface determinants identified by the HECA-452 mAb that support L-selectin-mediated leukocyte rolling (26). Untransfected 926 cells showed very weak HECA-452 mAb reactivity, while 926-FtVII cells expressed readily detected HECA-452-defined epitopes (Fig. 1). To examine the contribution of ICAM-1 expression to leukocyte/endothelial cell interactions, 926-ICAM-1 and 926-FtVII/ICAM-1 cells were generated, with cell lines expressing similar levels of HECA-452 Ag isolated by fluorescence-based cell sorting (Fig. 1). Similarly, 926-ICAM-1 and 926-FtVII/ICAM-1 cells were isolated that expressed ICAM-1 levels similar to those induced on 926 cells by TNF-α for 6 h (Fig. 1). HECA-452 mAb reactivity, while 926-FtVII cells expressed readily detected HECA-452-decorated epitopes (Fig. 1). To examine ICAM-1 expression by transfected 926 cells was stable during the course of these experiments.

ICAM-1 expression by 926-FtVII cells augments lymphocyte rolling

The extent to which ICAM-1 expression influences L-selectin-mediated rolling was determined by analyzing lymphocyte interactions with monolayers of 926 cells using a parallel plate flow chamber under physiologic shear flow. Neither 926 nor 926-ICAM-1 monolayers supported lymphocyte rolling (Fig. 2A). By contrast, significant numbers of lymphocytes rolled on 926-Ft-VII cells (Fig. 2A). Transfection of 926-FtVII cells with ICAM-1 increased lymphocyte rolling by 68% (p < 0.01). Anti-L-selectin mAb treatment completely abrogated lymphocyte rolling on 926-FtVII cells (data not shown) and 926-FtVII/ICAM-1 cells (Fig. 2A). Furthermore, lymphocyte rolling on 926-FtVII/ICAM-1 cells was reduced to the levels observed for 926-FtVII cells following treatment with an anti-CD18 mAb (p < 0.01; Fig. 2A). Thus, ICAM-1 alone does not support lymphocyte rolling under conditions of physiologic shear stress, but does enhance L-selectin-dependent rolling.

To determine whether increased lymphocyte rolling across 926-FtVII/ICAM-1 cells resulted from enhanced initial tether formation or the stabilization of rolling interactions, the frequency of initial contacts between lymphocytes and 926 cell lines was quantified. Tether formation was defined as a drop in lymphocyte velocity significantly <526 μm/s, the theoretical velocity of a free-flowing lymphocyte in this system (39). Tethering was assessed as lymphocytes first entered the flow chamber to exclude previously rolling cells. Lymphocytes formed similar numbers of contacts with 926-FtVII and 926-FtVII/ICAM-1 cells over a 10-s period, but did not interact with either 926 or 926-ICAM-1 monolayers (Fig. 2B). However, lymphocytes rolled ~26% slower on 926-FtVII/ICAM-1 cells than on 926-FtVII cells, with median rolling velocities of 235 and 174 μm/s, respectively (Fig. 2C; p < 0.001). Lymphocyte rolling was maximal at a shear stress of 1.5 dynes/cm<sup>2</sup> across both 926-FtVII and 926-FtVII/ICAM-1 monolayers (Fig. 2D). Importantly, 926-FtVII/ICAM-1 cells supported a significantly higher frequency of lymphocyte rolling than 926-FtVII cells at each shear stress, with a similar percent increase at all shear stresses examined. No lymphocyte rolling was detected on 926-ICAM-1 monolayers at any shear stress tested (Fig. 2D). Thus, the increase in lymphocyte rolling observed on 926-FtVII/ICAM-1 cells resulted from ICAM-1 stabilization of lymphocyte rolling after initial tether formation.
CD18 expression regulates lymphocyte rolling on vascular endothelial cells

To determine whether CD18 integrin interactions with ICAM-1 influence lymphocyte rolling, lymphocytes from CD18<sup>b<sup>hypo</sup> mice that have significantly reduced CD18 expression (~15% of wild-type levels; data not shown) (28) were assessed using in vitro flow chamber assays. Lymphocytes from CD18-deficient mice were not used for these assays because these mice develop spontaneous skin ulceration, chronic dermatitis, elevated leukocyte counts, and splenomegaly resulting from spontaneous infections and subacute inflammatory disorders (42). In contrast, CD18<sup>b<sub>hypo</sub></sup> mice do not suffer from these conditions (28), and no significant difference in L-selectin expression was found between lymphocytes from wild-type and CD18<sup>b<sub>hypo</sub></sup> mice (mean fluorescence intensity ± SEM, 107 ± 15 and 118 ± 16, respectively; Fig. 4A). PLN and MLN lymphocytes were used for the flow chamber assays because the frequency of T cells is higher in these tissues than in spleen (43), and T cells express L-selectin at higher levels than B cells (36). A mixture of lymphocytes from PLN and MLN was used in all assays because the frequency of rolling lymphocytes from each tissue was not significantly different (data not shown).

Consistent with human and mouse lymphocytes expressing equal levels of cell surface L-selectin (44), wild-type and CD18<sup>b<sub>hypo</sub></sup> mouse lymphocytes rolled on 926-FtVII cells (Fig. 4B) at levels similar to those of human lymphocytes (Fig. 2A). Also similar to human lymphocytes, significantly more wild-type mouse lymphocytes rolled on 926-FtVII/ICAM-1 cells than 926-FtVII cells (95% increase; p < 0.05). In addition, rolling velocities on 926-FtVII/ICAM-1 cells were similar for human and wild-type mouse lymphocytes (Figs. 2C and 4D). By contrast, CD18<sup>b<sub>hypo</sub></sup> lymphocytes rolled ~48% faster on 926-FtVII/ICAM-1 cells compared with wild-type lymphocytes (median velocity, 211 vs 143 μm/s, respectively; p < 0.001; Fig. 4D). That wild-type and CD18<sup>b<sub>hypo</sub></sup> mouse lymphocytes rolled at similar frequencies on 926-FtVII monolayers indicates that ICAM-2 expression by 926-FtVII cells appears predominantly attributable to CD18/ICAM-1 interactions.

Since lymphocyte subsets express cell surface L-selectin over a wide range of levels, the effect of decreased L-selectin expression...
on lymphocyte rolling was examined. Lymphocytes from L-selectin \(^{-/-}\) mice express L-selectin at 50% of wild-type levels (29), but showed a 66% reduction in rolling on 926-FtVII cells compared with wild-type lymphocytes (\(p < 0.05\); Fig. 4B). Although L-selectin \(^{-/-}\) lymphocytes did roll at significantly higher frequency than 926-FtVII/ICAM-1 cells (by 91%; \(p < 0.05\)), their rolling velocity was increased by 70% with ICAM-1 expression (by 140%) and by 52% with CD18 expression (by 140%; \(p < 0.001\); Fig. 4D). Thus, both CD18 integrins and ICAM-1 contribute to the frequency and quality of normal human and mouse lymphocyte rolling, with L-selectin surface density also regulating rolling frequencies and velocities.

**ICAM-1 expression mediates neutrophil adhesion on 926-FtVII cells**

Neutrophils did not roll on 926 or 926-ICAM-1 monolayers, but rolled on 926-FtVII and 926-FtVII/ICAM-1 monolayers (Fig. 5A). When neutrophil/endothelial cell interactions were measured on 926-FtVII and 926-FtVII/ICAM-1 cells over a 10-min period, the number of rolling neutrophils increased over the first 2 min and then remained stable (Fig. 5B). As with lymphocytes, rolling was completely blocked by anti-L-selectin mAb treatment. With increased ICAM-1 expression on 926-FtVII/ICAM-1 monolayers, significant numbers of adherent neutrophils accumulated in linear clusters (Fig. 5C), with the number of adherent neutrophils increasing linearly over time (Fig. 5B). In the absence of rolling,
Neutrophils did not adhere to 926 or 926-ICAM-1 monolayers at 1.85 dyne/cm² of shear stress (Fig. 5C). When neutrophils were treated with either an anti-L-selectin or anti-CD18 mAb, firm adhesion was blocked by >95% (Fig. 5C; p < 0.01). Thus, the expression of HECA-452-defined epitopes or increased ICAM-1 alone was unable to support firm adhesion, but ICAM-1 expression in combination with L-selectin facilitated human neutrophil/endothelial cell interactions that culminated in firm neutrophil adhesion.

To determine whether ICAM-1 expression influenced neutrophil rolling independently of firm adhesion, the rolling properties of individual neutrophils were examined. On the average, neutrophils rolled ~22% faster than lymphocytes on both 926-FtVII and 926-FtVII/ICAM-1 monolayers (Figs. 2C and 5D). Nonetheless, neutrophils rolled ~26% slower on 926-FtVII/ICAM-1 (median velocity, 211 μm/s) monolayers than on 926-FtVII (286 μm/s) monolayers (Fig. 5D; p < 0.001). At different shear stresses, the frequency of rolling neutrophils was highest at 1.25–1.5 dyne/cm², with no significant difference between 926-FtVII and 926-FtVII/ICAM-1 monolayers (Fig. 5E). Similarly, the highest rate of neutrophil firm adhesion to 926-FtVII/ICAM-1 monolayers was found at the same rates of shear. Interestingly, substantial numbers of neutrophils adhered to 926-FtVII/ICAM-1 monolayers at 0.5 dyne/cm² of shear stress, a point where few, if any, rolling neutrophils were observed (Fig. 5, E and F). However, few neutrophils adhered to 926-ICAM-1 monolayers in the absence of HECA-452 expression at shear stresses <1.0 dyne/cm² (Fig. 5, E and F). Thus, L-selectin and ICAM-1 contribute to neutrophil rolling through additive functions, although L-selectin-mediated interactions are still required for neutrophils to firmly adhere to 926-FtVII/ICAM-1 monolayers even under low shear stresses.

Neutrophils decelerate rolling velocity before firm adhesion

To examine how leukocytes initiate firm adhesion, rolling velocity and distance changes in individual neutrophils were traced on 926-FtVII/ICAM-1 monolayers before they firmly adhered. Typically, neutrophils tethered, started rolling, and decelerated their rolling velocity before arresting (Fig. 6). Firm adhesion usually occurred within 1–3 s of initial tether formation. Initial tether formation resulted in dramatic velocity reductions over subsecond time intervals. During rolling, the slopes of time-distance curves reflected the heterogeneity in rolling velocities observed for individual neutrophils (Fig. 5D). In general, neutrophils rolled for a short period with fluctuating velocities, but then rapidly decelerated and became firmly adherent (Fig. 6). Thus, the time-distance plots for individual leukocytes as they became adherent was best fit by an exponential model, as previously suggested for the transition from rolling to adhesion in vivo (46). Of interest, the rate of deceleration before adhesion varied significantly between individual leukocytes, with both slow and rapid deceleration observed. Abrupt arrest without apparent deceleration was only observed for a minority of leukocytes (Fig. 6, E, F, and J), although this may actually reflect rapid deceleration that is not adequately represented over the 0.1-s intervals used for measurements. Nonetheless, the transition from a rolling to a firmly adherent leukocyte is not an instantaneous event, but represents a gradual transformation process occurring over seconds.

**CD18 integrins and ICAM-1 influence lymphocyte migration to PLN and MLN**

The migration of CD18<sup>−/−</sup> lymphocytes was assessed to determine whether decreased lymphocyte rolling observed during in vitro assays correlated with decreased lymphocyte migration to lymphoid tissues in vivo. Calcein-labeled splenocytes from wild-type or CD18<sup>−/−</sup> mice were mixed with an equal number of internal control PKH26-labeled wild-type splenocytes and injected into wild-type recipient mice. In short term migration assays (1 h), CD18<sup>−/−</sup> splenocyte migration to PLNs and MLNs was 43 and 34% lower than wild-type levels, respectively (Fig. 7A). By contrast, CD18<sup>−/−</sup> splenocytes were present at significantly higher levels in the blood during short term migration assays. In long term migration assays (48 h), CD18<sup>−/−</sup> splenocyte migration to PLNs and MLNs remained significantly lower by 38–40% compared with wild-type migration (Fig. 7A). However, CD18<sup>−/−</sup> and wild-type splenocytes migrated to Peyer’s patches and spleen at similar frequencies at both time points.

To determine whether CD18 integrin interactions with ICAM-1 influence lymphocyte migration, the in vivo migration of wild-type lymphocytes was assessed in both wild-type and ICAM-1<sup>−/−</sup> recipient mice. In short term migration assays, lymphocyte migration to PLNs was 53% lower in ICAM-1<sup>−/−</sup> recipient mice compared with wild-type controls (Fig. 7B). Transferred lymphocytes were present at significantly higher levels in the blood of ICAM-1<sup>−/−</sup> mice during short term migration assays (Fig. 7B). After 48 h, lymphocyte migration to PLNs remained reduced by 43% in ICAM-1<sup>−/−</sup> recipient mice relative to that in wild-type mice. However, loss of ICAM-1 expression did not influence lymphocyte migration to spleen, MLNs, or Peyer’s patches. Thus, both CD18 integrin and ICAM-1 expression are required for normal lymphocyte migration to PLN.

**Discussion**

The role of CD18/ICAM-1 interactions during L-selectin-mediated lymphocyte rolling was directly quantified in this study using a

**FIGURE 6.** Time-distance and time-velocity curves for neutrophils tethering, rolling, and firmly adhering to 926-FtVII/ICAM-1 cell monolayers. A–J, Tracings for 10 representative neutrophils are shown from four independent experiments at 0.1-s intervals. Dotted lines indicate the theoretical velocity (526 μm/s) of a leukocyte not interacting with the endothelial cell surface.
CD18/ICAM-1 INTERACTIONS MODIFY LEUKOCYTE ROLLING VELOCITIES

FIGURE 7. Short and long term lymphocyte migration in vivo. A, Calcein-labeled splenocytes from wild-type or CD18+/+/ mice were mixed with an equal number of internal control PKH26-labeled wild-type splenocytes and injected into wild-type recipient mice. After 1 or 48 h, lymphocytes isolated from PLN, MLN, Peyer’s patches (PP), spleen (SPL), and blood (BL) were analyzed by two-color fluorescence cytometry. The ratio of calcein-labeled test cells to PKH26-labeled internal control cells within each tissue (Ri) was normalized by dividing by the injected ratio of calcein-labeled test cells to PKH26-labeled control cells (Ri) to generate R/R, ratios as described in Materials and Methods. Values represent the mean ± SEM of R/R, ratios obtained in three or four independent experiments, * Differences between CD18+/+ and wild-type splenocytes were significant (p < 0.05). B, Calcein-labeled lymphocytes were injected into wild-type or ICAM-1−/− recipient mice and were isolated 1 or 48 h later. The percentage of calcein-labeled lymphocytes isolated from each tissue was determined by fluorescence cytometry. The number of immigrated cells present within each tissue was then calculated from individual tissue cell counts. Values represent the mean ± SEM of results from 4–14 individual experiments. *, Differences between ICAM-1−/− and wild-type mice were significant (p < 0.05).

A series of 926 endothelial cell lines expressing L-selectin ligands and/or ICAM-1. 926 cell expression of ICAM-1 at levels comparable to those found on cytokine-activated endothelial cells (Fig. 1) was insufficient to support lymphocyte tether formation (Fig. 2B), but significantly enhanced the frequency and decreased the velocity of lymphocytes rolling on 926-FvII cells over a range of physiological shear stresses (Fig. 2, A, C, and D). Importantly, decreased lymphocyte rolling velocities on 926-FvII/ICAM-1 cell monolayers was not restricted to a subset of lymphocytes since the decrease in rolling velocities was proportional for both fast and slow rolling lymphocytes (Figs. 2C and 4D). Similar results were obtained when mouse lymphocyte rolling was assessed on 926 cell lines. Specifically, lymphocytes from CD18+/+ mice rolled less efficiently than those from wild-type mice on 926-FvII/ICAM-1 cells (Fig. 4). That the initial tether frequency of lymphocytes interacting with endothelial cell monolayers was not increased by ICAM-1 expression on 926-FvII cells, but lymphocyte rolling velocities were significantly reduced, suggests that ICAM-1 expression functions to stabilize lymphocyte rolling. These results are consistent with the significantly increased leukocyte rolling velocities observed in ICAM-1−/− mice during trauma- and TNF-α-induced inflammation (17) and with increased leukocyte rolling velocities reported in P-selectin/ICAM-1−/− mice compared with P-selectin−/− mice (20). As β2 integrin interactions with ICAM-1 alone did not promote lymphocyte tether formation or rolling (Fig. 2, A and B) even under reduced shear stress (Fig. 2D), ICAM-1 function during lymphocyte rolling supplements molecular interactions between other adhesion molecule pairs, such as L-selectin.

ICAM-1 expression also facilitated neutrophil rolling on 926-FvII cells by reducing rolling velocities (Fig. 5D). Neutrophils rolled on 926-FvII and 926-FvII/ICAM-1 monolayers at significantly faster rates than lymphocytes (Figs. 2 and 5), which is consistent with neutrophils rolling faster than lymphocytes on plate-bound CD34 (47). This difference did not result from L-selectin endoproteolytic release during neutrophil isolation (data not shown), but may result from the fact that neutrophils are larger than lymphocytes, while both neutrophils and lymphocytes express L-selectin at similar overall levels (48). Remarkably, the majority of neutrophils became firmly adherent within 3 s of initial tether formation (Fig. 6). The current concepts of leukocyte adhesion dictate that neutrophils require appropriate stimuli to up-regulate β2 integrin avidity and initiate firm adhesion (49, 50). The source of this stimulus in the in vitro assay used for this study is being assessed, but could result from signaling through L-selectin (32, 51, 52), low affinity CD18 interactions inducing integrin activation, low level neutrophil activation during isolation and shear stress, or the presence of appropriate chemokines that activate LFA-1 and promote firm adhesion of rolling cells. Nonetheless, the number of rolling neutrophils on 926-FvII/ICAM-1 monolayers was not significantly different from the number on 926-FvII cells. This is probably due to the rapid transition from rolling to firm adhesion on 926-FvII/ICAM-1 monolayers under flow, but not on either 926-FvII or 926-ICAM-1 cells (Figs. 5C and 6). That 926-ICAM-1 cells supported low levels of neutrophil firm adhesion at flow rates of 0.5 dyne/cm² in the near absence of L-selectin-mediated rolling (Fig. 5, E and F) is consistent with reduced shear stresses leading to CD18-dependent, selectin-independent leukocyte adhesion (16, 50). However, the number of adherent neutrophils on FvII/ICAM-1 cells was far greater than that on 926-ICAM-1 cells at low and high flow rates (Fig. 5, C and F). Neutrophil adhesion was also blocked equally by anti-L-selectin or anti-CD18 mAbs (Fig. 5C). Thus, ICAM-1 engagement contributes significantly to both rolling and firm adhesion, but does not mediate efficient tether formation even under conditions when selectin-mediated rolling is barely detected.

These studies reinforce a close correlation between decreased leukocyte rolling velocities and increased frequencies of rolling cells (Figs. 2, 4, and 5). However, considerable heterogeneity in overall rolling velocities remained between individual leukocytes (Figs. 2 and 4–6) and rolling velocities fluctuated considerably during the time that individual neutrophils rolled (Fig. 6). Despite their relative homogeneity in adhesion molecule expression, there were 10-fold differences in the expression of HECA-452 Ag and ICAM-1 expression by transfected 926 cells between the highest and lowest Ag-expressing cells (Fig. 1). In addition, L-selectin site density and oligomerization correlate closely with leukocyte migration and rolling velocities (40). In vivo, a 50% reduction in L-selectin expression density results in an ~70% decrease in short term migration to peripheral lymphoid tissues (36). In vitro, rolling of lymphocytes from L-selectin−/− mice was also decreased by ~70%, and increased ICAM-1 expression on 926-FvII cells did not compensate for the reduced L-selectin expression (Fig. 4B). Likewise, increased surface ICAM-1 expression on 926-FvII cells enhanced the frequency of rolling lymphocytes with similar contributions at both high and low shear stresses (Fig. 2D). CD18 deficiency in vivo leads to increased leukocyte rolling velocities that are most prominent at high shear stresses (53), with loss of...
either LFA-1 or Mac-1 alone also increasing rolling velocities (22). It is therefore likely that leukocyte rolling velocities on 926-FVIIICAM-1 cells were influenced by interactions of ICAM-1 with both LFA-1 and Mac-1. Thereby, ICAM-1 and L-selectin site densities and topological arrangements are likely to critically influence the dynamics of most leukocyte interactions with endothelial cells.

Encounters with chemoattractants or chemokines induces cellular activation in rolling leukocytes that results in integrin up-regulation and rolling arrest in vitro and in vivo (54). However, a recent issue of debate is whether neutrophils undergo instantaneous arrest upon chemokine encounter as occurs with lymphocytes (55) or whether there is a gradual deceleration process resulting from increasing integrin adhesiveness (46). When individual neutrophils were assessed for changes in their rolling velocities on 926 cell monolayers over time, most neutrophils behaved similarly following an initial capture event that resulted in rolling and rapid firm adhesion (<3 s; Fig. 6). Some neutrophils rapidly decelerated and firmly adhered within 1 s, while some gradually decelerated and finally adhered. That most of the time-distance curves were exponential immediately before neutrophil adhesion suggests a continual deceleration of rolling leukocytes that precedes final arrest and firm adhesion. Similar observations have recently been made for leukocyte arrest during cytokine-dependent inflammation in vivo, although the transition time from fast to slow rolling before firm adhesion was manifest over several minutes (46). Undoubtedly, the deceleration rate and its duration will be directly proportional to the biological activity and local concentrations of stimuli that up-regulate integrin affinities for ligands. Thereby, ICAM-1 engagement by β2 integrins may facilitate early selectin-mediated rolling, but may also decelerate rolling velocities as integrins transition from a low affinity to a high affinity state that results in firm adhesion.

The finding that ICAM-1 and CD18 integrins enhance lymphocyte rolling on vascular endothelial cells suggests that β2 integrins also contribute to leukocyte migration to peripheral lymphoid tissues. In support of this concept, the migration of CD18<sup>−/−</sup> spleenocytes to PLN was reduced by 40–43%, and migration to MLN was reduced by 34–38% in both short and long term migration assays (Fig. 7A). Migration of wild-type lymphocytes to PLNs was decreased by 43–53% in ICAM-1<sup>−/−</sup> recipient mice, with no significant difference found in numbers of lymphocytes migrating to MLNs (Fig. 7B). These results corroborate those obtained using LFA-1-deficient lymphocytes (56, 57), although the previously reported effects of LFA-1 deficiency on lymphocyte migration to PLN, MLN, and Peyer’s patches are much more pronounced than those found in this study using lymphocytes from CD18<sup>−/−</sup> mice or ICAM-1<sup>−/−</sup> recipient mice. One potential explanation is that relatively small changes in cell surface L-selectin expression levels have dramatic effects on lymphocyte rolling (Fig. 4, B and D) and in vivo migration (36). The expression level of cell surface L-selectin on leukocytes from L-selectin<sup>−/−</sup> mice has not been described, although they do not develop spontaneous skin lesions or infections (58, 59). An interesting finding of the present study was that wild-type lymphocytes migrated normally into MLNs of ICAM-1<sup>−/−</sup> mice, while CD18<sup>−/−</sup> lymphocytes demonstrated significantly reduced migration to MLN. There are several potential explanations for this difference in migration. LFA-1 interacts not only with ICAM-1, but also with ICAM-2, ICAM-3, and junctional adhesion molecule-1 (33, 60, 61). However, the present finding that ICAM-2 did not contribute significantly to lymphocyte rolling in vitro (Fig. 4) is consistent with LFA-1 interactions with ICAM-1 being less efficient than those with ICAM-1 (62) and with lymphocyte migration being unaffected by ICAM-2 deficiency (63). However, the contributions of other integrins and their ligands to lymphocyte migration to MLNs may be affected by the loss of CD18 integrins (64–66). Nonetheless, the current data suggest that CD18 integrin interactions with ICAM-1 contribute to the efficiency of lymphocyte rolling in venules of peripheral lymphoid tissues in addition to influencing lymphocyte adhesion.

Since the in vitro assay system used in the current study examines L-selectin and CD18/ICAM-1 function in isolation, it can be concluded that adhesive interactions between the β2 integrins and ICAM-1 contribute directly to leukocyte rolling. This reinforces our original conclusion that cell surface ICAM-1 expression reduces leukocyte rolling velocities on vascular endothelium in vivo (17). That rolling velocities of all cells were influenced by ICAM-1 expression and that leukocyte rolling over a range of shear stress was similarly affected indicate that this is a general property of CD18/ICAM-1 expression and is not limited to specific leukocyte subsets or conditions of shear stress or rolling velocities. Moreover, the current assay system is conducted under defined hemodynamic conditions and shear stresses, varying factors that have to be compensated for when using in vivo systems. The current assay system thereby provides a mechanism for future studies determining how other adhesion receptor pairs contribute to leukocyte/endothelial interactions under shear flow.

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References


