P-Selectin, L-Selectin, and α_4 Integrin Have Distinct Roles in Eosinophil Tethering and Arrest on Vascular Endothelial Cells Under Physiological Flow Conditions[1]

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The adhesive interactions of eosinophils with purified E-, P-, and L-selectins; vascular cell adhesion molecule-1 molecule; and HUVEC were examined in shear flow. Compared with neutrophils, eosinophils showed markedly less binding to E-selectin, but significantly stronger avidity for P-selectin. Both cell types showed a similar level of tethering and rolling on L-selectin. Eosinophils tethered and arrested abruptly on vascular cell adhesion molecule-1. However, some of the tethers were detached within several seconds; this was prevented by stimulation with eotaxin. Eosinophils also showed immediate arrest on HUVEC stimulated with 100 U/ml TNF-α for 6 h. Treatment with L-selectin mAb decreased eosinophil accumulation on the HUVEC by abrogating secondary tethers through interactions between flowing and attached eosinophils. mAb to P-selectin but not to E-selectin strongly inhibited primary tethers and accumulation of eosinophils. mAb to the integrin α_4 subunit inhibited arrest, induced rolling or detachment of tethered eosinophils, and resulted in partial reduction of eosinophil accumulation. mAb to the integrin β_2 subunit had only a slight effect, whereas treatment with mAb to the integrin α_4 and β_2 subunits together abolished rolling interactions as well as arrest, and thus almost totally inhibited eosinophil accumulation. Our data indicate that P-selectin, but not E-selectin, is directly involved in eosinophil tethering on inflammatory endothelium while L-selectin mainly mediates intereosinophil interaction. VLA-4 has a crucial role in eosinophil arrest, and arrest is enhanced by exposure to chemoattractants. *The Journal of Immunology,* **1997, 159: 3929–3939.**

Although a minor population among circulating blood leukocytes, eosinophils often predominate in tissues with chronic allergic inflammation, such as reactive airways, allergic rhinitis, or atopic skin reactions (1, 2). Numerous studies have proposed mechanisms to facilitate the selective recruitment of eosinophils into these pathologic tissues (3, 4). Leukocyte adhesion to local vascular endothelium is an essential process for leukocyte emigration and may be regulated by differential expression of adhesion molecules, chemoattractants, and their receptors. Binding to local endothelium may be divided into two steps: initial tethering of a cell in vascular flow followed by rolling, and development of firm adhesion (5, 6).

Firm adhesion is mainly mediated by integrins. In distinct contrast to neutrophils, eosinophils express very late Ag 4 (VLA-4)[3] ($\alpha_4\beta_1$), and studies with blocking mAb in static conditions have revealed that both VLA-4 and β_2 integrins mediate eosinophil adhesion to cytokine-activated endothelial cells (HUVEC), by binding to vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule-1, respectively (7–11). Furthermore, α_4 integrins contribute to rolling interactions in vivo (12). These results suggest that the expression of VCAM-1 in local endothelium may be one important mechanism for selective recruitment of eosinophils.

Local production of chemoattractants may be another important factor in selective leukocyte emigration, since chemoattractants activate integrin adhesive functions and stimulate directional migration of leukocytes across endothelium (6). Recent studies have shown that C-C chemokines, including RANTES (13–15), monocyte chemoattractant protein-3, monocyte chemoattractant protein-4 (16, 17), and eotaxin (18, 19) are potent and selective chemoattractants for eosinophils with high specificity and are abundantly expressed in tissues with allergic diseases.

Molecular mechanisms mediating the initial step of eosinophil tethering and rolling on endothelial cells have not been fully characterized. In vitro flow assays using purified molecules have revealed that E-selectin (20, 21), P-selectin (22), and VCAM-1 (23–26), all of which can be induced on endothelial cell surfaces by inflammatory mediators, support tethering and rolling of neutrophils or mononuclear cell in the presence of shear force. L-selectin has been shown not only to be involved in lymphocyte homing to lymphoid organs but also to be utilized in leukocyte infiltration to inflamed tissue, and has been proposed to recognize L-selectin ligands induced on cytokine-stimulated endothelial cells in vitro and in the microvasculature in vivo (27–30). However, several in vitro studies have failed to detect an involvement of L-selectin in the tethering of T cells on HUVEC activated with IL-1 or TNF-α (23, 31). Recent studies have shown that tethering of neutrophils and monocytes through L-selectin can be divided into two different categories: direct tethering on the underlying substrate (primary tethers), and cells accumulated downstream of previously adherent neutrophils after interactions between flowing leukocytes and previously adherent leukocytes (secondary tether). These result in accumulation of newly adherent cells downstream of previously adherent cells in a pattern of cells termed "strings" (32, 33). In other

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[3] Abbreviations used in this paper: VLA-4, very late antigen 4; VCAM-1, vascular cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1.

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studies it has been shown that neutrophils, monocytes, and eosinophils, but not human blood T lymphocytes, express L-selectin ligands as well as L-selectin, accounting for interleukocyte interactions in shear flow (34–38).

Previous studies on eosinophils have shown that purified eosinophils bind both to immobilized E-selectins (39) and P-selectins (40) in static conditions, although binding to E-selectin is less than observed with neutrophils, probably because of the lower expression of sialylated Lewis^x on eosinophils. In rotational adhesion assays, Stamper-Woodruff assays, and in mesentery, L-selectin (12, 41) and P-selectin (42) have been reported to be involved in eosinophil adhesion to endothelial cells. In flow assays, eosinophils have been found to roll less efficiently than neutrophils on E-selectin (43), and to roll on P-selectin with higher avidity than neutrophils (44). In this study, we use an in vitro flow chamber assay to analyze the interaction of purified eosinophils with cultured endothelial cells as well as with purified P-selectin, E-selectin, L-selectin, and VCAM-1. We demonstrate that eosinophils show unique adhesive interactions with these molecules compared with neutrophils.

Materials and Methods

Reagents and Abs

E-selectin was immunopurified from E-selectin-transfected CHO cells (CHO-E), using mAb BB11 (45) (a gift from Dr. R. Lobb, Biogen, Cambridge, MA) affinity chromatography. P-selectin, purified from platelets (46) was kindly provided by Dr. R. McEver (University of Oklahoma). L-selectin was immunopurified from human peripheral lymphocytes using mAb DREG56 (a gift from Dr. T. K. Kishimoto, Boehringer-Ingelheim, Ridgefield, CT) affinity chromatography (47). Soluble VCAM-1 was provided by Dr. R. Lobb, Biogen (Cambridge, MA) (48). Human eotaxin was chemically synthesized by Dr. Ian Clark-Lewis and purified by high pressure liquid chromatography (19). TNF-α was purchased from Genzyme (Cambridge, MA). mAbs HP2/1 to integrin α_4 (Amac, Westbrook, ME), DREG56, TS1/18 to integrin β_2 (49), BB11 (45) and W6/32 to MHC class I were used as purified IgG. mAbs to P-selectin HDPG2/3 (blocking) and HPDG2/1 (nonblocking) were kindly provided by Dr. Dale Cummings (Genetics Institute, Cambridge, MA) (50). For blocking experiments, leukocyte-directed mAbs were incubated with cells at 20 μg/ml in 0.1 ml HBSS with 10 mM HEPES, pH 7.4, for 30 min on ice and then diluted to 1 ml of assay media (HBSS with 10 mM HEPES supplemented with 2 mM Ca^{2+}, 1 mM Mg^{2+}, and 0.2% human serum albumin, pH 7.4) and used for experiments (final concentration of 2.0 μg/ml). HUVEC-directed mAbs were added to HUVEC cultures at a concentration of 40 μg/ml and incubated for 30 min at 37°C before experiments were performed.

Cell purification

Granulocytes were isolated from citrate-anticoagulated whole blood from normal volunteers by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of the granulocyte cell pellet to remove RBCs (51). Granulocytes, washed twice with PBS containing 1% BSA and 5 mM EDTA, were used as the input neutrophil population (>92–96% neutrophils). Identical results were obtained using eosinophils purified with Percoll gradients, in which a hypotonic lysis step was not used. Eosinophils were purified from granulocytes by negative immunomagnetic selection using anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec, Sunnyvale, CA) (52). Isolates routinely contained >99% eosinophils with viability >95% by trypan blue exclusion.

Preparation of protein substrate or HUVEC monolayer

Purified P-, E-, or L-selectin in octylglucoside detergent were dissolved in PBS buffered with 10 mM bicarbonate (pH 8.0) and immediately spotted on polystyrene plates (Lab-Tek; Nunc, Naperville, IL) for 2 h, washed three times with PBS, and blocked with 20 μg/ml human serum albumin fraction V (Calbiochem, La Jolla, CA) in PBS for 2 h at 37°C and incubated for an additional 20 h at 4°C. Soluble VCAM-1 was diluted into 50 mM Tris (HCl, pH 8.0) and coated as described above. HUVEC were isolated (53) and cultured in six-well dishes coated with 40 μg/ml type-1 collagen in medium 199 (M199) supplemented with 15% FCS and 50 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA) and 100 μg/ml heparin (Sigma Chemical Co., St. Louis, MO). After reaching confluence, the HUVEC monolayers were cultured for another 6 h with or without 00 U/ml (10 μg/ml) TNF-α. For all experiments, primary or first passaged HUVEC were used. Increased expression by TNF-α of E-selectin and P-selectin on these cells has previously been characterized (31, 54).

Laminar flow assay

The plastic plate (90 mm × 90 mm) on which adhesion molecules were adsorbed was assembled as the lower wall of a parallel-plate flow chamber, and mounted on the stage of an inverted phase-contrast microscope and viewed with a ×20 objective (22, 55). In experiments using HUVEC, the flow chamber was mounted on a HUVEC monolayer in one well (35 mm in diameter) of a six-well plate, and the monolayer was used for just one flow experiment. Before placement of the chamber on the HUVEC monolayer, care was taken to eliminate air bubbles in the flow chambers, since the flowing bubbles easily disrupted the HUVEC monolayer. All flow experiments were performed at 37°C using a thermostatted air chamber on the microscope. Wall shear stress was calculated as previously described (22). A cell suspension (10^6/ml) was perfused through the flow chamber using an automated syringe pump (Harvard Apparatus, Natick, MA) attached to the outlet side. Chemoattractants were added to cells immediately before infusion, and cells reached the point of microscopic observation within a few seconds. Cells interacting with the substrate during flow were quantitated by analysis of images videotaped with a TEC-470 CCD video camera (Optronics, Goleta, CA) and Hi-8 Sony CVD-1000 recorder. All experiments were scored by two independent observers, one of whom was blinded.

Evaluation of primary tethering, secondary tethering, and accumulation and detached, rolling, or arrested cells

Tethered cells were defined as cells that maintained an adhesive interaction with the substrate for at least 1 s. Tethering efficiency was measured as the number of cells that formed tethers over the initial 30 s of infusion, when leukocyte tethering occurred mostly through direct interactions between flowing leukocyte and underlying substrate, i.e., when most tethers were primary. For the analysis of post-tethering behavior, the movement of each tethered cell was observed for 30 s after it first tethered. Cells detached in the flow stream in this period were counted as detached cells. Among the cells that remained adherent, cells that were displaced less than or more than 1 cell diameter for 30 s were defined as arrested and rolling cells, respectively. For exact quantification of primary tethers on HUVEC, adherent cells present in the field were first identified at the 1.0-min time point, then the videotape was played backward in slow motion and only the cells that tethered directly to HUVEC without a preceding leukocyte-leukocyte interaction were counted as primary tethers. Total cell accumulation was defined as the number of cells that arrested or remained rolling at the end of the 3-min observation period. p values were calculated by paired Student's t test, and the differences with $p < 0.05$ were considered to be significant.

Results

Eosinophil tethering and rolling on E-selectin and P-selectin

Tethering and rolling of freshly isolated eosinophils on E-selectin and P-selectin in physiologic shear flow was compared with that of neutrophils under the same conditions (Fig. 1). Selectin-coating conditions were adjusted to result in nearly equal tethering of neutrophils to E- and P-selectin over the shear range tested. Eosinophils formed substantially fewer tethers to E-selectin than neutrophils at all shear levels and only a few eosinophils tethered at more than 1.5 dyn/cm^2 (Fig. 1*A*). In contrast, eosinophils bound to P-selectin somewhat more efficiently than neutrophils over a wide range of shear rates (Fig. 1*B*), and the difference was significant at 2.0 dyn/cm^2 ($p < 0.05$, $n = 3$).

Although all the cells that tethered on purified E- and P-selectin were observed to roll in shear flow, the rolling velocity and shear resistance contrasted between neutrophils and eosinophils. In accordance with their lower tethering efficiency on E-selectin, eosinophils rolled on purified E-selectin with significantly faster speed than neutrophils over a range of wall shear stresses (Fig. 1*D*). By contrast, the rolling velocity of eosinophils on P-selectin

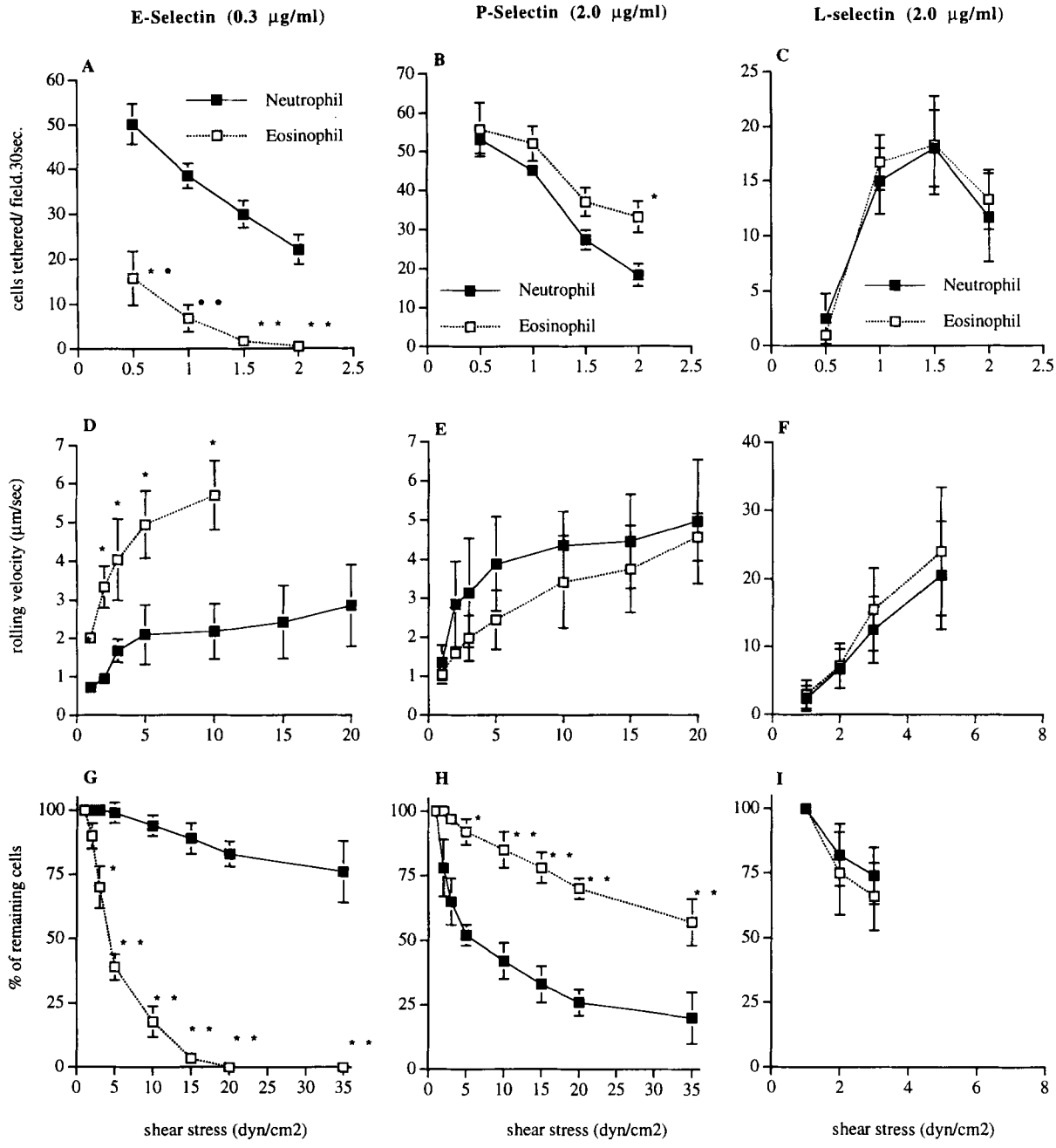

FIGURE 1. Adhesive interactions of eosinophils and neutrophils with E-selectin, P-selectin, and L-selectin substrates in shear flow. Tethering efficiency (*A–C*), rolling velocity (*D–F*), and detachment profile (*G–I*) of neutrophils (closed square) or eosinophils (open square) on substrates bearing E-, P-, and L-selectin. Purified, full-length E-selectin, P-selectin, and L-selectin at 0.3, 2.0, and 2.0 μg/ml, respectively, were adsorbed to plastic substrates. *A–C*, Cell suspensions (10^6/ml) were perfused in the flow chamber at the indicated wall shear stresses. The number of cells that tethered in the first 30 s was counted. Values show mean ± SD in three independent experiments (*A*, *B*) or in four different fields in two independent experiments (*C*). *D* to *F*, Cell suspensions (10^6/ml) were perfused at 0.5 dyn/cm^2 for 2 min (*D*, *E*) or at 1.0 dyn/cm^2 for 1 min (*F*), and then the shear was increased every 15 s. Rolling velocity was calculated for 12 to 20 cells in each shear condition in individual experiments and expressed as mean ± SD of 40 to 60 cells examined in two (*F*) or three (*D*, *E*) different experiments. *G–I*, In the same experiments as for *D* to *F*, the number of cells that remained rolling adherent in the field was counted at the end of each flow interval and expressed as the percentage cells adherent after tethering for 2 min at 0.5 dyn/cm^2 (*G–H*) or for 1 min at 1 dyn/cm^2 (*I*). In *F* and *I*, both cell types rolled quickly out of the field of view at shear stress of 3.0 to 5.0 dyn/cm^2, and values could not be accurately measured at higher shear. *, $p < 0.05$; **, $p < 0.01$, eosinophils compared with neutrophils.

was slightly slower than that of neutrophils, correlating with slightly higher tethering efficiency (Fig. 1*E*). The profile of resistance to detachment at increasing wall shear stress on E-selectin demonstrated that rolling adhesions of neutrophils were dramatically more stable than those of eosinophils (Fig. 1*G*). In contrast, on P-selectin, rolling adhesions of eosinophils were more shear resistant than those of neutrophils (Fig. 1*H*). The three different measures of selectin-mediated interactions in shear flow demonstrated that binding of eosinophils to E-selectin was dramatically less efficient and stable than of neutrophils, whereas binding of

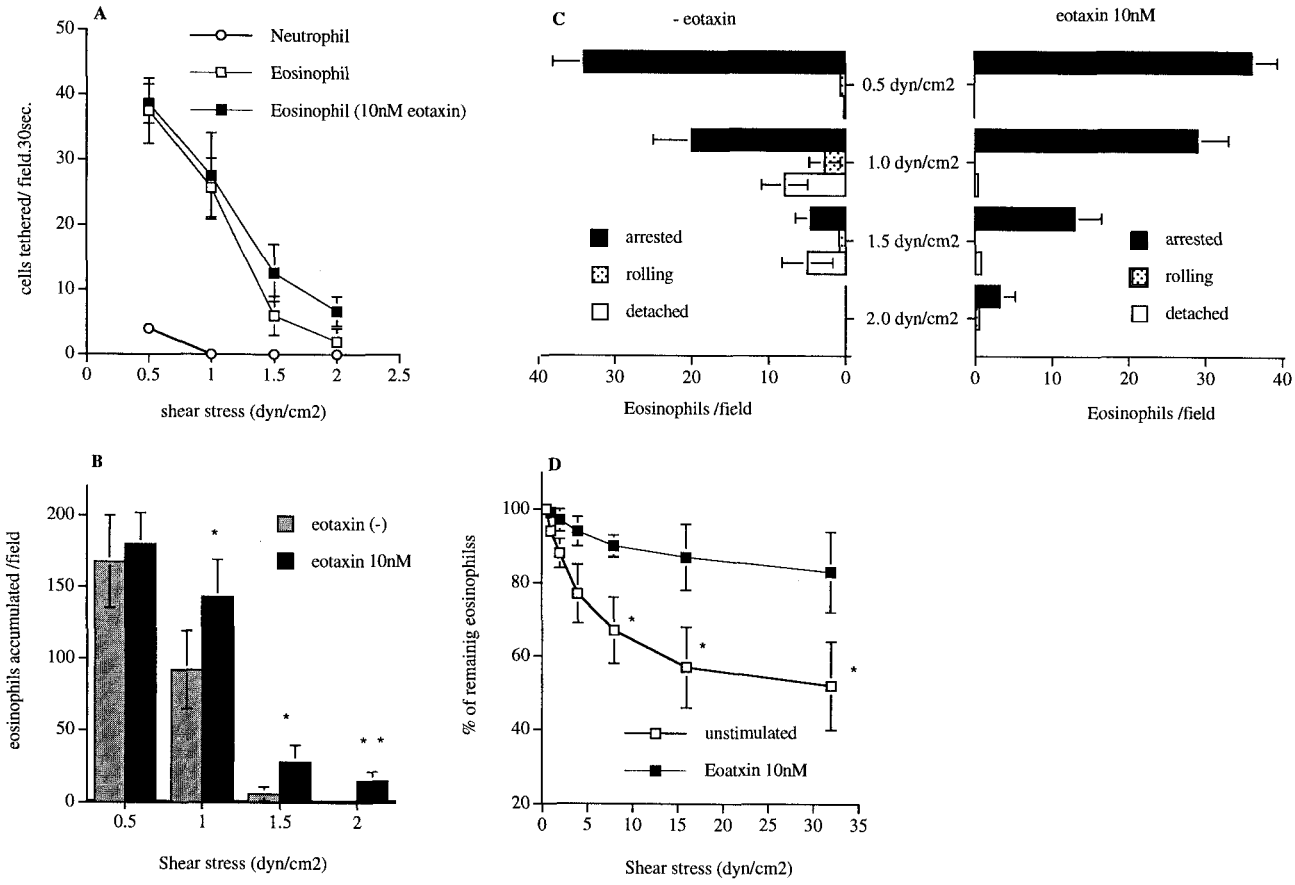

FIGURE 2. Eosinophil interaction with immobilized VCAM-1 and effect of eotaxin. Soluble VCAM-1 at 5.0 μg/ml was coated on plastic and eosinophil suspensions (10^6/ml) were perfused at the indicated wall shear stresses (*A–C*) or at 0.5 dyn/cm^2 for 2 min, and the shear was then increased every 15 s (*D*). Eotaxin was added to the eosinophil suspensions at a final concentration of 10 nM just before perfusion. Tethering efficiency (*A*) and total accumulation (*B*) were evaluated after the initial 30 s and at 3 min after the start of perfusion, respectively. *C*, Each tethered cell detected in *A* was carefully examined for 30 s after it tethered. Arrested, rolling, and detached cells were defined as described in *Materials and Methods*. *D*, Cells remaining after each shear step were calculated as percentage of the cells accumulated at the shear of 0.5 dyn/cm^2 for 2.0 min. Values represent mean ± SD in three independent experiments. *, $p < 0.05$; **, $p < 0.01$, between presence and absence of eotaxin.

eosinophils and neutrophils to P-selectin was more comparable, with eosinophil adhesion consistently more efficient.

Eosinophil interaction with L-selectin

In the flow experiments on purified P-selectin, we observed eosinophils in flow to accumulate preferentially downstream of previously tethered eosinophils, which resulted in the formation of strings as previously reported for neutrophils (32, 56). L-selectin ligands have been reported to be expressed on and are well characterized on neutrophils (34, 35, 37) and γδ T cells (57). L-selectin ligands are also expressed on eosinophils (37), but have not been characterized in detail or compared in efficiency to those on neutrophils. Therefore, we examined interactions with immobilized L-selectin purified from PBLs to test for differences in L-selectin ligand activities between neutrophils and eosinophils. In contrast to results on E-selectin and P-selectin, neutrophil and eosinophil tethering to purified L-selectin was far less efficient at 0.5 dyn/cm^2 than at shear stresses above 1.0 dyn/cm^2 (Fig. 1*C*). Thus, eosinophil rolling adhesions on L-selectin as substrate require a shear stress above a threshold value, as reported for neutrophils and T cells using an L-selectin ligand as the substrate (58, 59). Both eosinophils and neutrophils rolled on L-selectin with much faster velocity than on E- or P-selectins (Fig. 1*F*). However, no significant differences were observed between the two cell types in tethering efficiency, rolling velocity, shear resistance, or the shear threshold value required for tether formation (Fig. 1, *C*, *F*, and *I*).

Eosinophil interaction with VCAM-1

Since eosinophils highly express VLA-4 that can support tethering and rolling of PBL on VCAM-1 (25, 26), we examined the interaction of eosinophils with purified VCAM-1. Eosinophils, and not neutrophils, tethered and accumulated on plastic coated with 5 μg/ml of soluble VCAM-1 (Fig. 2*A*). The number of tethers (Fig. 2*A*) and amount of cell accumulation (Fig. 2*B*) decreased at shear stresses above 1.5 dyn/cm^2, in contrast to results with P-selectin and L-selectin (Fig. 1, *B* and *C*). Moreover, the behavior of cells after tethering on VCAM-1 was notably different from on selectins. More than 95% of tethered eosinophils arrested immediately after tethering on VCAM-1 at 0.5 dyn/cm^2, i.e., without an intervening period of rolling (Fig. 2*C*). Even at higher shear stress, most of the tethered eosinophils did not roll at 1 and 1.5 dyn/cm^2, and many of the nonrolling, as well as rolling, cells subsequently detached within 30 s after tethering (Fig. 2*C*). Some of the detached cells had tethered on VCAM-1 only transiently, while others showed jerky rolling for several seconds before detachment. When VCAM-1 was coated at higher concentrations, the percentage of detached cells at each shear decreased, but again the tethered cells mostly arrested abruptly, and few cells were observed to

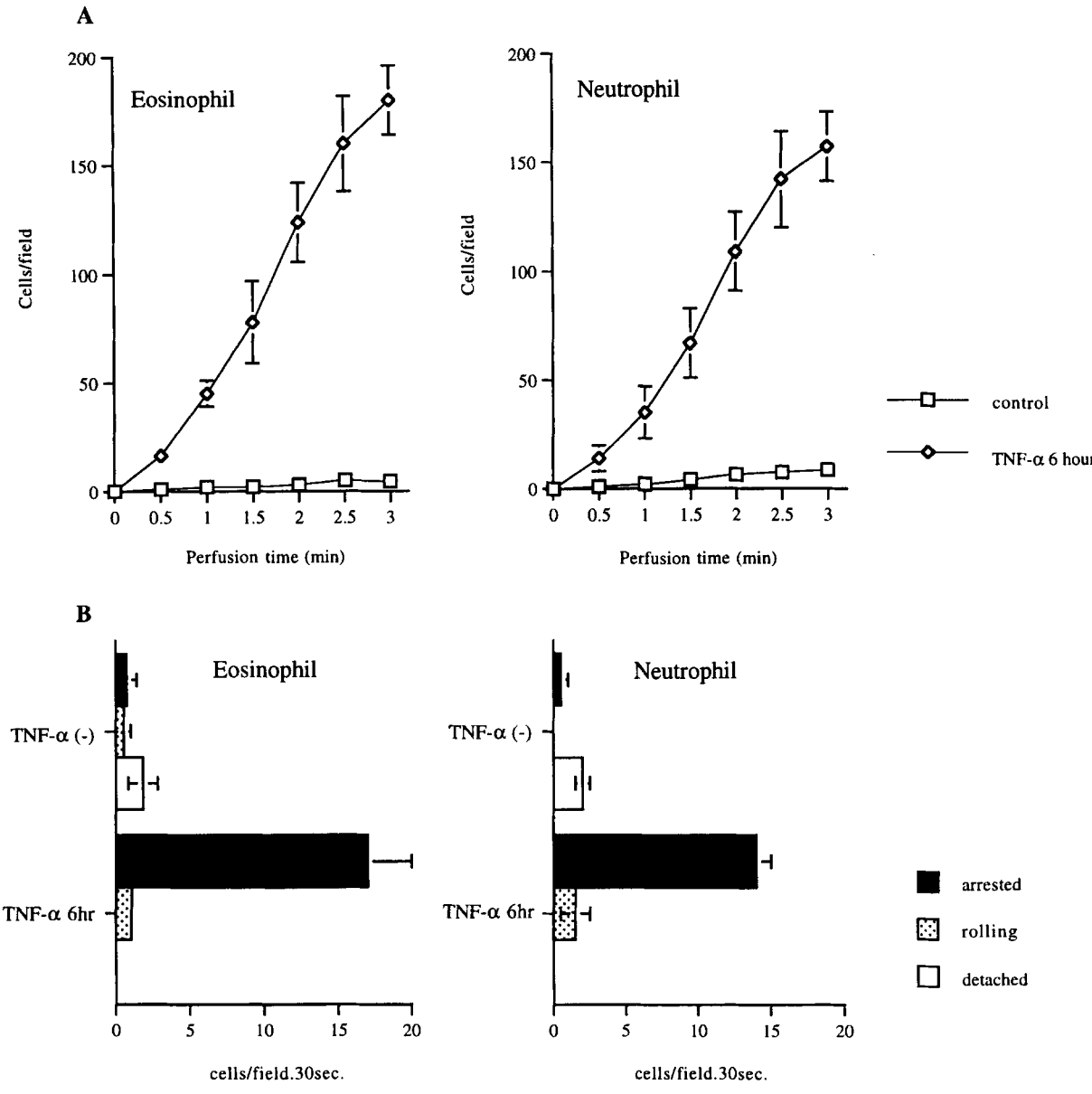

FIGURE 3. Accumulation (*A*) and tethering (*B*) of eosinophils or neutrophils on resting or TNF-α-activated HUVEC. Cell suspensions (10^6/ml) were perfused on confluent HUVEC cultured for 6 h with or without 100 U/ml TNF-α. *A*, Cell accumulation. *B*, Each cell tethered in the initial 30-s perfusion period was examined for a 30-s period after it tethered. Arrested, rolling, and detached cells were determined as described in Figure 2. Values show mean ± range of two different experiments.

roll on VCAM-1 under any experimental condition (data not shown).

Effect of the chemoattractant eotaxin on eosinophil interaction with VCAM-1

In the multistep model of leukocyte adhesion to local endothelium, chemoattractants are proposed to trigger firm adhesion by increasing integrin avidity (6, 22). Therefore, we examined the effect of eotaxin on eosinophil interaction with substrates bearing purified VCAM-1. Eotaxin, a member of the CC chemokine family that is a potent chemoattractant for eosinophils (18, 19), was added to eosinophils just before perfusion in the flow chamber at 10 nM, an optimal concentration in chemotaxis assays (19) (data not shown). Eotaxin slightly increased tethering efficiency to VCAM-1 at wall shear stresses above 1.5 dyn/cm^2, although this was not significant (Fig. 2*A*). However, total eosinophil accumulation was significantly increased at 1.0, 1.5, and 2.0 dyn/cm^2 (Fig. 2*B*). This was because the number of arrested cells was dramatically and statistically significantly increased by eotaxin at and above 1.0 dyn/cm^2 (Fig. 2*C*). Almost all eotaxin-stimulated eosinophils arrested immediately after tethering at all shear stresses examined (Fig. 2*C*). Resistance to detachment by increasing shear stresses was also significantly increased by eotaxin at 8 dyn/cm^2 and above (Fig. 2*D*).

Eosinophil interaction with cultured endothelial cells in flow

Interactions with endothelial cells were compared between eosinophils and neutrophils by perfusing the cells on HUVEC monolayers with or without stimulation by 100 U/ml TNF-α for 6 h. In both cell types, only a few cells attached on resting HUVEC, whereas large numbers of cells accumulated on TNF-α-stimulated HUVEC at a shear of 1.5 dyn/cm^2 (Fig. 3, *A* and *B*). Neutrophils and eosinophils showed similar kinetics in accumulation on the

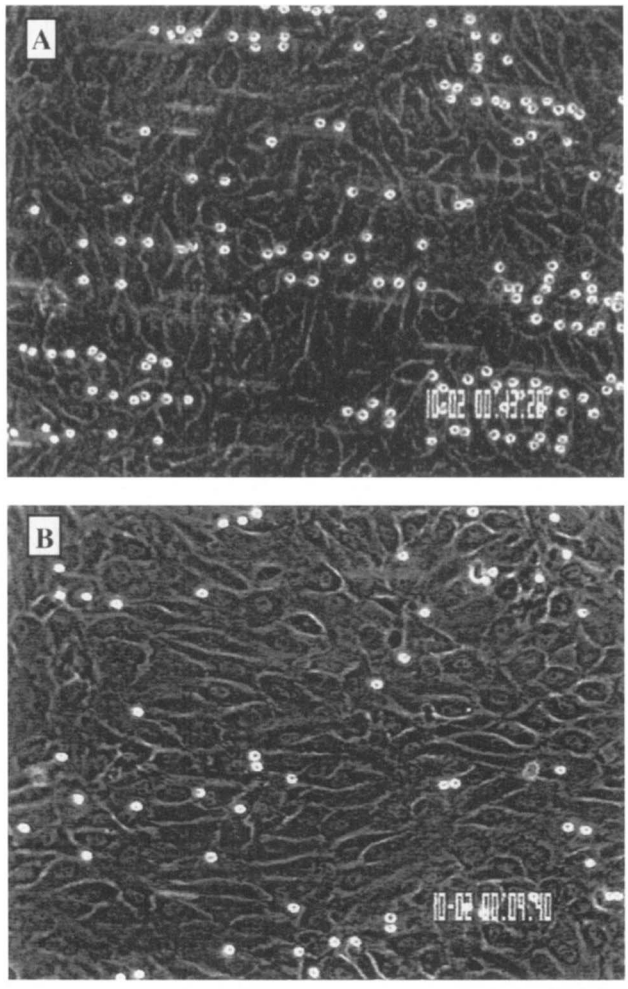

FIGURE 4. Pattern of eosinophil accumulation on activated HUVEC. *A*, Control eosinophils or *B*, DREG56-treated eosinophils were perfused at 1.5 dyn/cm^2 for 3 min on HUVEC stimulated with 100 U/ml TNF-α for 6 h.

HUVEC. For the early perfusion period, most of the tethers occurred randomly on the HUVEC monolayers; thereafter, more eosinophils (Fig. 4*A*) and neutrophils (not shown) accumulated downstream of previously attached cells, with formation of strings at the end of the perfusion (32, 33). In keeping with this, cell accumulation accelerated after 0.5 min (Fig. 3*A*). This suggests that interleukocyte interactions occurred and augmented cell accumulation at later time periods. The post-tethering behavior was examined for cells that attached in the initial 30-s period. Of the few cells tethered on resting HUVEC for this period, most detached within 30 s after tethering (Fig. 3*B*). By contrast, many more eosinophils and neutrophils tethered on activated HUVEC, and most of the tethered cells were immediately arrested (Fig. 3*B*). Although a few tethered cells rolled, all rolled for no more than a few cell diameters before arresting, and no cells detached during the observed period.

Blocking of eosinophil adhesion on TNF-α-stimulated HUVEC with mAbs to selectins

Treatment with mAb DREG56 to L-selectin markedly reduced eosinophil and neutrophil accumulation on TNF-α-stimulated HUVEC (Fig. 5, *right*). However, the number of primary tethers, i.e., cells that bound directly on the HUVEC surface without a preceding interleukocyte interaction, was minimally affected (Fig. 5, *left*). Furthermore, L-selectin mAb-treated eosinophils (Fig. 4*B*) and neutrophils (data not shown) accumulated with a random distribution on the HUVEC monolayer, with no strings observed. Treatment of HUVEC with mAb BB11 to E-selectin strongly decreased both primary tethers and accumulation of neutrophils (Fig. 5*A*) ($p < 0.01$, $n = 3$). The effect on both neutrophil primary tethers and total accumulation was in marked contrast to the effect of L-selectin mAb. BB11 further enhanced the inhibitory effect of DREG56 on neutrophil accumulation, when compared with DREG56 alone (Fig. 5*A*, *right*) ($p < 0.05$, $n = 3$). In marked contrast to its effect on neutrophils, BB11 showed no significant effect on eosinophil tethers on accumulation, whether used alone or together with DREG56 (Fig. 5*B*). However, the blocking HPDG2/3 mAb to P-selectin greatly decreased eosinophil primary tethers and accumulation (42 ± 4.7%, $p < 0.01$, $n = 3$, and 45 ± 8.3%, $p < 0.02$, $n = 3$, respectively). HPDG2/3 mAb also decreased neutrophil primary tethers (22 ± 3.4%, $p < 0.01$, $n = 3$) and accumulation (24 ± 2.1%, $p < 0.01$, $n = 3$), but to a lesser extent. The ratios of inhibition were significantly greater for eosinophils than neutrophils ($p < 0.05$ for tethering, $p < 0.01$ for accumulation, $n = 3$). In all these experiments, most of the tethered cells were immediately arrested on activated HUVEC, and the treatment with anti-selectin mAbs had no apparent effect on post-tethering behavior in either cell type.

Blocking of eosinophil adhesion on TNF-α-stimulated HUVEC with mAbs to integrins

Integrin function in eosinophil accumulation on HUVEC was examined with mAb HP2/1 to the α_4 and mAb TS1/18 to the leukocyte β_2 integrin subunits. Treatment with HP2/1 did not significantly affect the total number of tethers on TNF-α-stimulated HUVEC (the total of arrested, rolling, and detached cells, Fig. 6, *left*). However, treatment with α_4 mAb dramatically changed the post-tethering behavior of eosinophils. Instead of arresting, most of the HP2/1-treated eosinophils rolled on the HUVEC, with only 23% of the cells arrested. Moreover, about 30% of the eosinophils detached during the following 30 s. About half of the cells counted as rolling cells arrested permanently at later time points, while other cells continued rolling or "jumping" with intervening transient arrests for the entire period that could be observed. As a result, treatment with α_4 mAb HP2/1 decreased eosinophil accumulation by 44 ± 14% (Fig. 6, *right*) ($p < 0.05$, $n = 3$). The blocking mAb TS1/18 to the β_2 integrin subunit reduced eosinophil accumulation slightly, but not significantly (Fig. 6, *right*). Most of the TS1/18 mAb-treated cells arrested on the HUVEC, although some of the tethered cells were counted as rolling (Fig. 6, *left*). However, they rolled only for a few cell diameters before arresting. A striking inhibition was obtained when the TS1/18 mAb to β_2 and the HP2/1 mAb to α_4 were used together. The total number of tethers was reduced by 44 ± 6.1% (Fig. 6, *left*) ($p < 0.01$, $n = 3$, compared with HP2/1 or TS1/18 alone). Furthermore, the number of arrested and rolling cells was greatly decreased compared with treatment with either mAb alone, with most tethered cells subsequently detaching (Fig. 6, *left*). Thus, eosinophil accumulation was almost totally inhibited by combined treatment with HP2/1 and TS1/18 (Fig. 6, *right*) ($p < 0.001$, $n = 3$). As expected, HP2/1 had no effect on neutrophil tethering or accumulation on HUVEC, either alone or in combination with TS1/18 (data not shown).

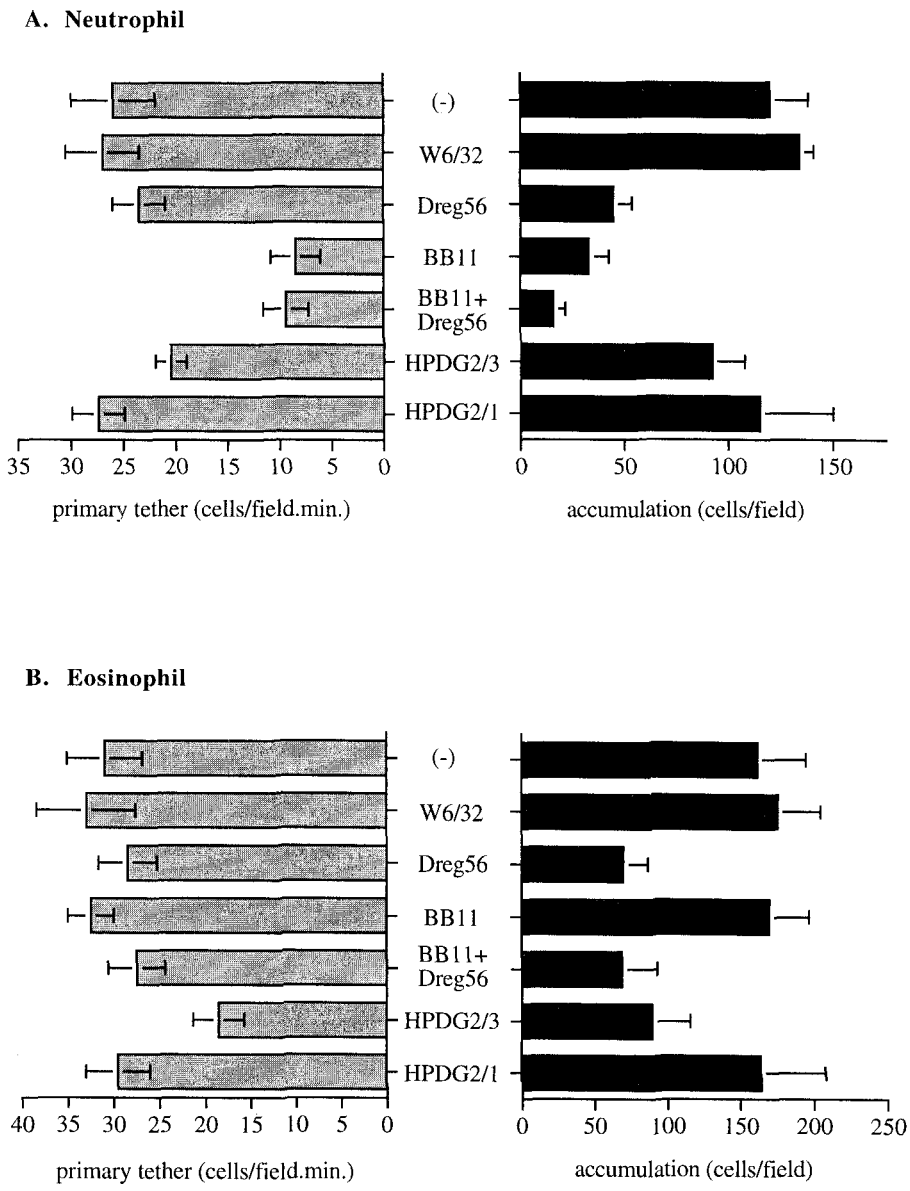

FIGURE 5. Effects of anti-selectin mAbs on primary tethers and total accumulation of neutrophils (*A*) or eosinophils (*B*) on HUVEC activated with 100 U/ml TNF-α for 6 h. Control cells or anti-L-selectin (DREG56)-treated cells were perfused on the HUVEC monolayer at 1.5 dyn/cm^2 for 3 min. mAb to HUVEC, BB11 (anti-E-selectin), HPDG2/3 (blocking mAb to P-selectin), and HPDG2/3 (nonblocking mAb to P-selectin) were directly added to HUVEC 30 min before perfusion. Accumulation was examined at the end of perfusion, and the number of primary tethers were counted for the initial 1.0 min of perfusion by close examination of videotape, as described in *Materials and Methods*. Each value represents mean ± SD of three independent experiments.

Discussion

Although there is substantial evidence that selectins and VLA-4 can mediate leukocyte rolling adhesion in shear flow, the role of these molecules and their relative importance in eosinophil interactions with endothelium in shear flow has not been clear. An in vivo study has shown that the number of eosinophils rolling on an IL-1-stimulated rabbit mesenteric vessel wall is partially decreased by mAbs to L-selectin or the α_4 integrin subunit (12). In this study, however, treatment of eosinophils with a combination of these two mAbs inhibited eosinophil rolling by about 50%, suggesting the existence of other adhesion pathways. Both E- and P-selectin have been shown to support neutrophil rolling in shear flow (20–22, 60). However, a previous static binding assay (39) and Stamper-Woodruff assay (42) have suggested that eosinophil binding to E-selectin is significantly weaker than neutrophil binding, because of the low expression of E-selectin ligands. Recently, it has been shown that mAb to E-selectin significantly inhibits the rolling interaction of neutrophils, but not of eosinophils, with IL-1-stimulated rabbit mesenteric venules (43), and that eosinophil influx to the pleural cavity is effectively inhibited by mAbs to P-selectin or L-selectin, but not by mAb to E-selectin in an LPS-induced mouse pleuritis model (61).

Consistent with previous studies, we found that eosinophils showed markedly less tethering efficiency, faster rolling velocity, and less resistance to detachment than neutrophils on purified E-selectin. However, we emphasize that the presence of shear stress strongly enhances the difference in E-selectin binding between neutrophils and eosinophils, and that very few eosinophils accumulated on E-selectin at physiologic shear rates for postcapillary venules (1.0 to ~30 dyn/cm^2).

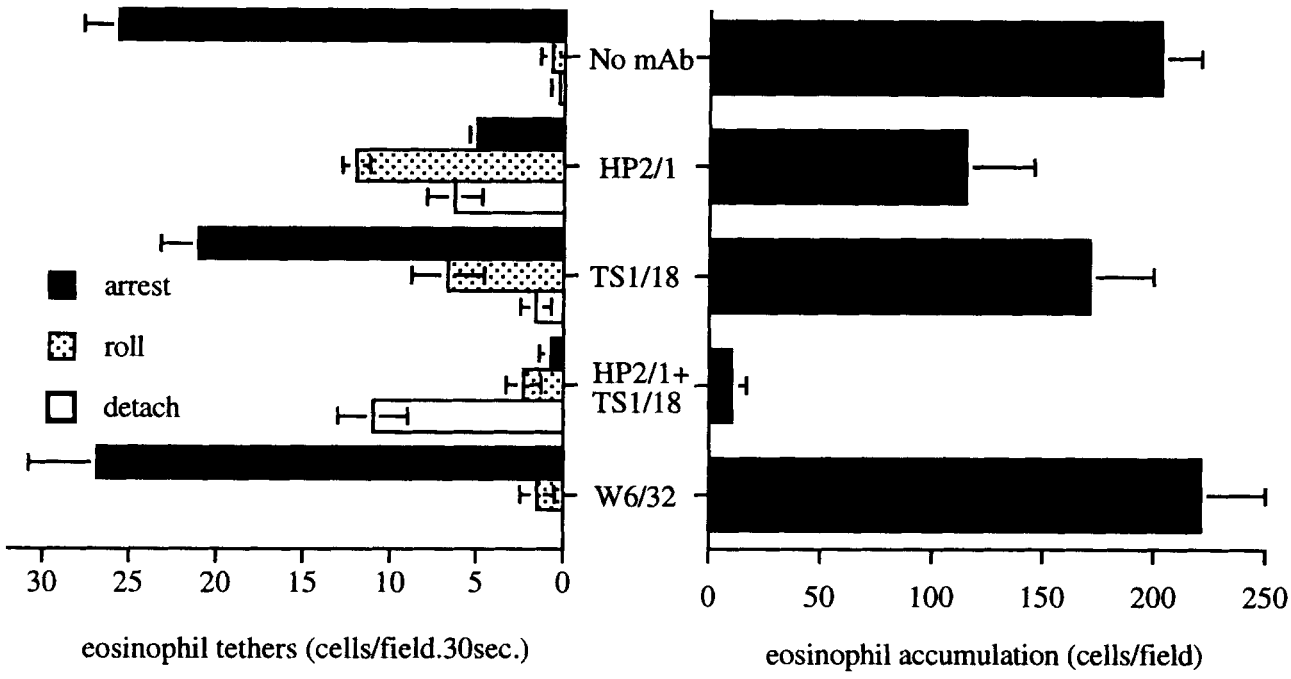

FIGURE 6. Effect of anti-integrin mAbs on eosinophil tethering, post-tethering behavior, and accumulation on HUVEC activated with TNF-α for 6 h. Eosinophils were pretreated with HP2/1 (anti-α_4) and/or TS1/18 (anti-β_2) and perfused on the HUVEC at 1.5 dyn/cm^2 for 3 min. Cells that tethered in the first 30 s were followed for 30 s after tethering, and cells that arrested, rolled, or detached were counted. Accumulation was measured at the end of the 3-min perfusion period. Each value shows mean ± SD of three independent experiments.

In contrast to the results on E-selectin, eosinophils and neutrophils were reported to bind to P-selectin nearly equivalently in a static assay (40). Our results are consistent with this, but also clearly show that eosinophils bind more avidly than neutrophils to P-selectin at relatively high shear. Consistent with this, the expression of PSGL-1, a major ligand for P-selectin, was higher in eosinophils than in neutrophils as examined by reactivity with a rabbit polyclonal Ab to PSGL-1 (Rb3302) (data not shown). This is in agreement with observations that eosinophils have higher avidity for P-selectin than neutrophils and that PSGL-1 on eosinophils is not only expressed at higher levels but also differs structurally from PSGL-1 on neutrophils (44).

Eosinophils tethered on purified VCAM-1 at lower shear stresses than on selectins. In contrast to the observations on E-, P-, and L-selectins, most of the tethered eosinophils did not roll, but immediately arrested on VCAM-1. In the presence of constant shear flow (1.0 dyn/cm^2 or above), some of the tethered eosinophils detached into the flow stream, but none of them showed persistent rolling on VCAM-1. In contrast, most purified PBL as well as K562 cells transfected with VLA-4 rolled on the same substrate (25) (data not shown). Purified monocytes also arrested immediately on immobilized VCAM-1 with only a few rolling cells (C. Weber, unpublished observations). The reason for this difference is not clear, but might be attributed to functional differences between α_4 integrins on lymphocytes on one hand and eosinophils and monocytes on the other. We obtained similar results using two different eosinophil preparation techniques; however, it cannot be excluded that eosinophils could have been partially activated in the course of purification. All the cells used in the flow assay retained a spherical shape and expressed L-selectin as highly as eosinophils before purification.

The VLA-4 molecule on resting eosinophils did not appear to be fully activated, because addition of 10 nM of eotaxin increased the resistance of tethered eosinophils to increasing shear stresses, and essentially all eotaxin-stimulated eosinophils that tethered subsequently arrested on VCAM-1 at all shears examined. This is consistent with our previous finding that the avidity of eosinophil VLA-4 can be rapidly enhanced by chemoattractant stimulation (62). Our findings demonstrate that α_4 integrins on circulating eosinophils can mediate transient arrest on VCAM-1 without preceding rolling, even in the absence of chemoattractant stimulation, whereas exposure with chemoattractant prevents detachment of tethered eosinophils and therefore enhances eosinophil accumulation under physiologic shear force.

On TNF-α-stimulated HUVEC at 1.5 dyn/cm^2, most of the tethered eosinophils showed abrupt arrest, similar to the results on VCAM-1. Blocking with mAbs to E-selectin and P-selectin further confirmed that P-selectin, but not E-selectin, was involved in eosinophil tethering. Together with our data on purified molecules and the results in previous reports (39, 42, 43, 61), it can be concluded that eosinophil tethering on endothelium is strongly dependent on P-selectin but not on E-selectin in physiologic shear conditions. Additionally, the finding that eosinophils have a higher avidity for P-selectin than neutrophils suggests that the selective induction on endothelial cells of P-selectin compared with E-selectin, as by thrombin or histamine (63, 64), may favor preferential eosinophil accumulation.

L-selectin has been reported to be involved in the recruitment of neutrophils as well as mononuclear cells on inflamed endothelium both in vitro (24, 27, 41, 65) and in vivo (28–30, 66, 67). L-selectin mAb has also been reported to inhibit eosinophil adhesive interactions with endothelial cells both in vitro (41) and in vivo (12). Consistent with these reports, more than half of eosinophil and neutrophil accumulation on TNF-α-stimulated HUVEC was blocked by L-selectin mAb in our study. However, recent studies have suggested that L-selectin on neutrophils does not bind directly to TNF-α-activated HUVEC, but binds to L-selectin ligands on neutrophils, and mainly mediates "secondary tethers" through

homotypic interactions between nonadherent and adherent neutrophils in flow (32, 56). We tested for any differences in L-selectin ligand activity between neutrophils and eosinophils, and found that eosinophils tethered and rolled on purified L-selectin equally to neutrophils, suggesting that eosinophils express L-selectin ligands at the same level. On TNF-activated HUVEC, eosinophils formed strings, which are evidence for interleukocyte interactions, and strings were totally abrogated by L-selectin mAb. Close observation of videotapes showed that treatment with L-selectin mAb had little if any effect on primary tethers on HUVEC. Inhibition of cell accumulation was mostly attributed to the abrogation of secondary tethers. Our results suggest that one important function of L-selectin in eosinophil recruitment is to mediate interleukocyte interactions, as previously discussed for neutrophils (32).

A mAb to the integrin α_4 subunit did not significantly reduce the tethering efficiency of eosinophils on TNF-α-stimulated HUVEC, but dramatically changed post-tethering behavior. This can be mainly attributed to the interaction of $\alpha_4\beta_1$ with VCAM-1 but may be partially dependent on $\alpha_4\beta_7$, since $\alpha_4\beta_7$ is expressed, although at lower levels than $\alpha_4\beta_1$, on eosinophils (68, 69). The inability of α_4 mAb to decrease eosinophil tethers on activated HUVEC is consistent with the finding that few eosinophils tethered on VCAM-1 at 1.5 dyn/cm^2, the wall shear stress used for the experiments on HUVEC. These data suggest that α_4 integrins do not have a major role in initial tethering of eosinophils in physiologic shear conditions.

By contrast to its lack of effect on tethering on activated HUVEC, mAb to α_4 strikingly inhibited arrest of eosinophils. In the presence of α_4 mAb, most tethered eosinophils showed subsequent rolling on stimulated HUVEC, and some were detached within a short time, resulting in a decrease in eosinophil accumulation by 44 $\pm$ 14%. A similar result has been described for monocytes (24, 54) and CD4$^+$ T cells (31) on cytokine-activated HUVEC, although it was not as marked as for eosinophils. From these data, we conclude that although α_4 integrins on eosinophils can effectively make bonds with VCAM-1 in shear flow, they do not have a major role in primary tethering on activated HUVEC. The principal role of α_4 integrins appears to be in arrest of eosinophils that are tethered by other molecules expressed on the HUVEC, such as P-selectin.

The rolling of α_4 mAb-treated eosinophils on HUVEC appeared to be mediated by P-selectin, since use of P-selectin mAb HPDG2/3 together with α_4 mAb further inhibited rolling interactions (data not shown). However, eosinophils treated with α_4 mAb alone rolled only for a short period, and about 30% of tethered eosinophils detached from the HUVEC surface within 30 s, suggesting that in contrast to the observation on purified molecules, P-selectin alone did not mediate persistent rolling and accumulation on the HUVEC surface. A portion of the rolling eosinophils arrested at later time points. This arrest was mostly dependent on β_2 integrins, because treatment with mAbs to β_2 and α_4 integrins together totally abrogated arrests and accumulation of eosinophils. However, α_4-mediated arrest usually predominates and occurs more immediately, since mAb to the β_2 integrin subunit alone has little effect on eosinophil arrest. Interestingly, almost all eosinophils treated with the combination of these two anti-integrin mAbs tethered only transiently, and rolling interactions as well as arrest formation were almost completely abolished. Moreover, the number of tethers was also significantly decreased by this treatment, although not affected by each mAb alone. This was an unexpected finding, because integrins are not considered to be involved in the initial tethering and rolling step. In this study, we defined tethered cells as the cells interacting with HUVEC for at least 1 s. Therefore, some shorter-lived transient tethers may be neglected by our measurements. However, our findings suggest that selectin-mediated interactions are not sufficient to support stable eosinophil rolling on stimulated endothelial cells, and that cooperation with α_4 and β_2 integrins are required for eosinophil accumulation by stabilizing rolling and enhancing arrest. A decrease in interleukocyte tethers through L-selectin because of a lack of firmly adherent leukocytes may also result from inhibition with mAb to α_4 and β_2 integrins and partially contribute to the effect. A caveat to the insufficiency of P-selectin in supporting rolling is that P-selectin expression declines with successive passages of HUVEC in vitro, and even though we have used HUVEC passaged only one to two times, P-selectin expression may be higher on endothelium in vivo.

Another interesting question is whether chemoattractants are involved in the step of eosinophil arrest on TNF-α-activated HUVEC. On purified VCAM-1, at 1.5 dyn/cm^2 some eosinophils detached within 30 s after tethering, and detachment was totally prevented by eotaxin stimulation. By contrast, almost all tethered eosinophils immediately arrested on TNF-α-stimulated HUVEC. Although it is still unknown whether TNF-α induces sufficient chemoattractant production for eosinophil activation, platelet activating factor (70) and RANTES (71) have been reported to be produced by TNF-α-stimulated HUVEC. In preliminary studies, we find that the supernatant of TNF-α-stimulated HUVEC is chemotactic for eosinophils and significantly increases eosinophil binding to purified intercellular adhesion molecule-1 and VCAM-1 in static conditions. This suggests that chemoattractants produced by activated endothelium may be involved in stimulation of eosinophil arrest through enhancement of integrin-mediated adhesion.

The expression of α_4 as well as β_2 integrins, L-selectin as well as L-selectin ligand, P-selectin ligand, and well-characterized receptors for chemoattractants, make eosinophils an interesting cell type for studying a wide range of steps in leukocyte accumulation on activated endothelium. Our findings show that eosinophils use P-selectin, but not E-selectin, for primary tethering on activated endothelium. L-selectin is important in mediating secondary tethers through interleukocyte interactions. Integrin α_4 has a minor role in initial tethers but plays a central role in arrest formation. Integrin α_4 and VCAM-1 interaction mediates immediate arrest of eosinophils without prerequisite rolling. Chemoattractant stimulation may further strengthen this adhesion pathway and can augment eosinophil accumulation in shear flow. β_2 integrins contribute to eosinophil accumulation and appear to act later than the α_4 integrin.

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References

1. Gleich, G. J., and C. R. Adolphson. 1986. The eosinophilic leukocyte: structure and function. *Adv. Immunol. 39:177.*
2. Bousquet, J., P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, and F.-B. Michel. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med. 323:1033.*
3. Resnick, M. B., and P. F. Weller. 1993. Mechanisms of eosinophil recruitment. *Am. J. Respir. Cell Mol. Biol. 8:349.*
4. Kita, H., and G. J. Gleich. 1996. Chemokines active on eosinophils: potential roles in allergic inflammation. *J. Exp. Med. 183:2421.*
5. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell 67:1033.*
6. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multi-step paradigm. *Cell 76:301.*
7. Weller, P. F., T. H. Rand, S. E. Goelz, G. Chi-Rosso, and R. R. Lobb. 1991. Human eosinophil adherence to vascular endothelium mediated by binding to vascular cell adhesion molecule 1 and endothelial leukocyte adhesion molecule 1. *Proc. Natl. Acad. Sci. USA 88:7430.*

8. Dobrina, A., R. Menegazzi, T. M. Carlos, E. Nardon, R. Cramer, T. Zacchi, J. M. Harlan, and P. Patriarca. 1991. Mechanisms of eosinophil adherence to cultured vascular endothelial cells: eosinophils bind to the cytokine-induced endothelial ligand vascular cell adhesion molecule-1 via the very late activation antigen-4 integrin receptor. *J. Clin. Invest. 88:20.*
9. Bochner, B. S., F. W. Luscinskas, M. A. Gimbrone, Jr., W. Newman, S. A. Sterbinsky, C. P. Derse-Anthony, D. Klunk, and R. P. Schleimer. 1991. Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. *J. Exp. Med. 173:1553.*
10. Walsh, G. M., J-J. Mermod, A. Hartnell, A. B. Kay, and A. J. Wardlaw. 1991. Human eosinophil, but not neutrophil, adherence to IL-1-stimulated human umbilical vascular endothelial cell is $\alpha_4\beta_1$ (very late antigen-4) dependent. *J. Immunol. 146:3419.*
11. Schleimer, R. P., S. A. Sterbinsky, J. Kaiser, C. A. Bickel, D. A. Klunk, K. Tomioka, W. Newman, F. W. Luscinskas, M. A. Gimbrone, Jr., B. W. McIntrye, and B. S. Bochner. 1992. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: association with expression of VCAM-1. *J. Immunol. 148:1086.*
12. Sriramarao, P., U. H. von Andrian, E. C. Butcher, M. A. Bourdon, and D. H. Broide. 1994. L-selectin and very late antigen-4 integrin promote eosinophil rolling at physiological shear rates in vivo. *J. Immunol. 153:4238.*
13. Rot, A., M. Krieger, T. Brunner, S. C. Bischoff, T. J. Schall, and C. A. Dahinden. 1992. RANTES and macrophage inflammatory protein 1α induce the migration and activation of normal human eosinophil granulocytes. *J. Exp. Med. 176:1489.*
14. Kameyoshi, Y., A. Dörschner, A. I. Mallet, E. Christophers, and J.-M. Schröder. 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med. 176:587.*
15. Ebisawa, M., T. Yamada, C. Bickel, D. Klunk, and R. P. Schleimer. 1994. Eosinophil transendothelial migration induced by cytokines. III. Effect of the chemokine RANTES. *J. Immunol. 153:2153.*
16. Dahinden, C. A., T. Geiser, T. Brunner, V. von Tscharner, D. Caput, P. Ferrara, A. Minty, and M. Baggiolini. 1994. Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J. Exp. Med. 179:751.*
17. Uguccioni, M., P. Loetscher, U. Forssmann, B. Dewald, H. Li, S. H. Lima, Y. Li, B. Kreider, G. Garotta, M. Thelen, and M. Baggiolini. 1996. Monocyte chemotactic protein 4 (MCP-4), a novel structural and functional analogue of MCP-3 and eotaxin. *J. Exp. Med. 183:2379.*
18. Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med. 179:881.*
19. Ponath, P. D., S. Qin, D. J. Ringler, I. Clark-Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J.-A. Gonzalo, W. Newman, J.-C. Gutierrez-Ramos, and C. R. Mackay. 1996. Cloning of the human eosinophil chemoattractant, eotaxin: expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J. Clin. Invest. 97:604.*
20. Lawrence, M. B., and T. A. Springer. 1993. Neutrophils roll on E-selectin. *J. Immunol. 151:6338.*
21. Abbassi, O., T. K. Kishimoto, L. V. McIntire, D. C. Anderson, and C. W. Smith. 1993. E-selectin supports neutrophil rolling in vitro under conditions of flow. *J. Clin. Invest. 92:2719.*
22. Lawrence, M. B., and T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell 65:859.*
23. Jones, D. A., L. V. McIntire, C. W. Smith, and L. J. Picker. 1994. A two-step adhesion cascade for T cell/endothelial cell interactions under flow conditions. *J. Clin. Invest. 94:2443.*
24. Luscinskas, F. W., G. S. Kansas, H. Ding, P. Pizcueta, B. E. Schleiffenbaum, T. F. Tedder, and M. A. Gimbrone, Jr. 1994. Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, β_1-integrins, and β_2-integrins. *J. Cell Biol. 125:1417.*
25. Alon, R., P. D. Kassner, M. W. Carr, E. B. Finger, M. E. Hemler, and T. A. Springer. 1995. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J. Cell Biol. 128:1243.*
26. Berlin, C., R. F. Bargatze, U. H. von Andrian, M. C. Szabo, S. R. Hasslen, R. D. Nelson, E. L. Berg, S. L. Erlandsen, and E. C. Butcher. 1995. α_4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell 80:413.*
27. Spertini, O., F. W. Luscinskas, G. S. Kansas, J. M. Munro, J. D. Griffin, M. A. Gimbrone, Jr., and T. F. Tedder. 1991. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J. Immunol. 147:2565.*
28. von Andrian, U. H., P. Hansell, J. D. Chambers, E. M. Berger, I. T. Filho, E. C. Butcher, and K. E. Arfors. 1992. L-selectin function is required for β_2-integrin-mediated neutrophil adhesion at physiological shear rates in vivo. *Am. J. Physiol. 263:H1034.*
29. von Andrian, U. H., J. D. Chambers, E. L. Berg, S. A. Michie, D. A. Brown, D. Karolak, L. Ramezani, E. M. Berger, K. E. Arfors, and E. C. Butcher. 1993. L-selectin mediates neutrophil rolling in inflamed venules through sialyl Lewis^x-dependent and -independent recognition pathways. *Blood 82:182.*
30. Ley, K., T. F. Tedder, and G. S. Kansas. 1993. L-selectin can mediate leukocyte rolling in untreated mesenteric venules in vivo independent of E- or P-selectin. *Blood 82:1632.*
31. Luscinskas, F. W., H. Ding, and A. H. Lichtman. 1995. P-selectin and vascular cell adhesion molecule 1 mediate rolling and arrest, respectively, of CD4$^+$ T lymphocytes on tumor necrosis factor α-activated vascular endothelium under flow. *J. Exp. Med. 181:1179.*
32. Alon, R., R. C. Fuhlbrigge, E. B. Finger, and T. A. Springer. 1996. Interactions through L-selectin between leukocytes and adherent leukocytes nucleate rolling adhesions on selectins and VCAM-1 in shear flow. *J. Cell Biol. 135:849.*
33. Walcheck, B., J. Kahn, J. M. Fisher, B. B. Wang, R. S. Fisk, D. G. Payan, C. Feehan, R. Betageri, K. Darlak, A. F. Spatola, and T. K. Kishimoto. 1996. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. *Nature 380:720.*
34. Simon, S. I., Y. P. Rochon, E. B. Lynam, C. W. Smith, D. C. Anderson, and L. A. Sklar. 1993. β_2-integrin and L-selectin are obligatory receptors in neutrophil aggregation. *Blood 82:1097.*
35. Bargatze, R. F., S. Kurk, E. C. Butcher, and M. A. Jutila. 1994. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J. Exp. Med. 180:1785.*
36. Oxley, S. M., and R. Sackstein. 1994. Detection of an L-selectin ligand on a hematopoietic progenitor cell line. *Blood 84:3299.*
37. Fuhlbrigge, R. C., R. Alon, K. D. Puri, J. B. Lowe, and T. A. Springer. 1996. Sialylated, fucosylated ligands for L-selectin expressed on leukocytes mediate tethering and rolling adhesions in physiologic flow conditions. *J. Cell Biol. 135:837.*
38. Spertini, O., A-S. Cordey, N. Monai, L. Giuffre, and M. Schapira. 1996. P-selectin glycoprotein ligand 1 is a ligand for L-selectin on neutrophils, monocytes, and CD34$^+$ hematopoietic progenitor cells. *J. Cell Biol. 135:523.*
39. Bochner, B. S., S. A. Sterbinsky, C. A. Bickel, S. Werfel, M. Wein, and W. Newman. 1994. Differences between human eosinophils and neutrophils in the function and expression of sialic acid-containing counterligands for E-selectin. *J. Immunol. 152:774.*
40. Wein, M., S. A. Sterbinsky, C. A. Bickel, R. P. Schleimer, and B. S. Bochner. 1995. Comparison of human eosinophil and neutrophil ligands for P-selectin: ligands for P-selectin differ from those for E-selectin. *Am. J. Respir. Cell Mol. Biol. 12:315.*
41. Knol, E. F., F. Tackey, T. F. Tedder, D. A. Klunk, C. A. Bickel, S. A. Sterbinsky, and B. S. Bochner. 1994. Comparison of human eosinophil and neutrophil adhesion to endothelial cells under nonstatic conditions: role of L-selectin. *J. Immunol. 153:2161.*
42. Symon, F. A., G. M. Walsh, S. R. Watson, and A. J. Wardlaw. 1994. Eosinophil adhesion to nasal polyp endothelium is P-selectin-dependent. *J. Exp. Med. 180:371.*
43. Sriramarao, P., C. R. Norton, P. Borgstrom, R. G. DiScipio, B. A. Wolitzky, and D. H. Broide. 1996. E-selectin preferentially supports neutrophil but not eosinophil rolling under conditions of flow in vitro and in vivo. *J. Immunol. 157:4672.*
44. Symon, F. A., M. B. Lawrence, M. L. Williamson, G. M. Walsh, S. R. Watson, and A. J. Wardlaw. 1996. Functional and structural characterization of the eosinophil P-selectin ligand. *J. Immunol. 157:1711.*
45. Lobb, R. R., G. Chi-Rosso, D. R. Leone, M. D. Rosa, S. Bixler, B. M. Newman, S. Luhowskyj, C. D. Benjamin, I. R. Dougas, S. E. Goelz, C. Hession, and E. P. Chow. 1991. Expression and functional characterization of a soluble form of endothelial-leukocyte adhesion molecule 1. *J. Immunol. 147:124.*
46. Patel, K. D., K. L. Moore, M. U. Nollert, and R. P. McEver. 1995. Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. *J. Clin. Invest. 96:1887.*
47. Picker, L. J., R. A. Warnock, A. R. Burns, C. M. Doerschuk, E. L. Berg, and E. C. Butcher. 1991. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell 66:921.*
48. Pepinsky, B., C. Hession, L.-L. Chen, P. Moy, L. Burkly, A. Jakubowski, E. P. Chow, C. Benjamin, G. Chi-Rosso, S. Luhowskyj, and R. Lobb. 1992. Structure/function studies on vascular cell adhesion molecule-1. *J. Biol. Chem. 267:17820.*
49. Sanchez-Madrid, F., J. Nagy, E. Robbins, P. Simon, and T. A. Springer. 1983. A human leukocyte differentiation antigen family with distinct alpha subunits and a common beta subunit: the lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. *J. Exp. Med. 158:1785.*
50. Sako, D., X.-J. Chang, K. M. Barone, G. Vachino, H. M. White, G. Shaw, G. M. Veldman, K. M. Bean, T. J. Ahern, B. Furie, D. A. Cumming, and G. R. Larsen. 1993. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell 75:1179.*
51. Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer. 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J. Cell Biol. 111:3129.*
52. Hansel, T. T., J. D. Pound, D. Pilling, G. D. Kitas, M. Salmon, T. A. Gentle, S. S. Lee, and R. A. Thompson. 1989. Purification of human blood eosinophils by negative selection using immunomagnetic beads. *J. Immunol. Methods 122:97.*
53. Gimbrone, M. A., Jr. 1976. Culture of vascular endothelium. *Prog. Hemost. Thromb. 3:1.*
54. Luscinskas, F. W., H. Ding, P. Tan, D. Cumming, T. F. Tedder, and M. E. Gerritsen. 1996. L- and P-selectins, but not CD49d (VLA-4) integrins, mediate monocyte initial attachment to TNF-α-activated vascular endothelium under flow in vitro. *J. Immunol. 156:326.*
55. Jones, D. A., O. Abbassi, L. V. McIntire, R. P. McEver, and C. W. Smith. 1993. P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophys. J. 65:1560.*
56. Walcheck, B., K. L. Moore, R. P. McEver, and T. K. Kishimoto. 1996. Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1: a mechanism that amplifies initial leukocyte accumulation on P-selectin in vitro. *J. Clin. Invest. 98:1081.*

57. Jutila, M. A., and S. Kurk. 1996. Analysis of bovine γδ T cell interactions with E-, P-, and L-selectin: characterization of lymphocyte on lymphocyte rolling and the effects of *O*-glycoprotease. *J. Immunol. 156:289.*
58. Finger, E. B., K. D. Puri, R. Alon, M. B. Lawrence, U. H. von Andrian, and T. A. Springer. 1996. Adhesion through L-selectin requires a threshold hydrodynamic shear. *Nature 379:266.*
59. Lawrence, M. B., G. S. Kansas, E. J. Kunkel, and K. Ley. 1997. Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). *J. Cell Biol. 136:717.*
60. Olofsson, A. M., K.-E. Arfors, L. Ramezani, B. A. Wolitzky, E. C. Butcher, and U. H. von Andrian. 1994. E-selectin mediates leukocyte rolling in interleukin-1 treated rabbit mesentery venules. *Blood 84:2749.*
61. Henriques, G. M. O., J. M. Miotla, R. S. B. Cordeiro, B. A. Wolitzky, S. T. Woolley, and P. G. Hellewell. 1996. Selectins mediate eosinophil recruitment in vivo: a comparison with their role in neutrophil influx. *Blood 87:5297.*
62. Weber, C., J. Kitayama, and T. A. Springer. 1996. Differential regulation of β_1- and β_2-integrin avidity by chemoattractants in eosinophils. *Proc. Natl. Acad. Sci. USA 93:10939.*
63. McEver, R. P., J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton. 1989. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest. 84(1):92.*
64. Bevilacqua, M. P., and R. M. Nelson. 1993. Selectins. *J. Clin. Invest. 91:379.*
65. Spertini, O., F. W. Luscinskas, M. A. Gimbrone, Jr., and T. F. Tedder. 1992. Monocyte attachment to activated human vascular endothelium in vitro is mediated by leukocyte adhesion molecule-1 (L-selectin) under nonstatic conditions. *J. Exp. Med. 175:1789.*
66. Ley, K., P. Gaehtgens, C. Fennie, M. S. Singer, L. A. Lasky, and S. D. Rosen. 1991. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood 77:2553.*
67. von Andrian, U. H., J. D. Chambers, L. M. McEvoy, R. F. Bargatze, K. E. Arfors, and E. C. Butcher. 1991. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte β_2 integrins in vivo. *Proc. Natl. Acad. Sci. USA 88:7538.*
68. Erle, D. J., M. J. Briskin, E. C. Butcher, A. Garcia-Pardo, A. I. Lazarovits, and M. Tidswell. 1994. Expression and function of the MAdCAM-1 receptor, integrin $\alpha_4\beta_7$, on human leukocytes. *J. Immunol. 153:517.*
69. Walsh, G. M., F. A. Symon, A. I. Lazarovits, and A. J. Wardlaw. 1996. Integrin $\alpha_4\beta_7$ mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin. *Immunology 89:112.*
70. Camussi, G., F. Bussolino, G. Salvidio, and C. Baglioni. 1987. Tumor necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *J. Exp. Med. 166:1390.*
71. Marfaing-Koka, A., O. Devergne, G. Gorgone, A. Portier, T. J. Schall, P. Galanaud, and D. Emilie. 1995. Regulation of the production of the RANTES chemokine by endothelial cells. *J. Immunol. 154:1870.*