

Interactions of Human α/β and γ/δ T Lymphocyte Subsets in Shear Flow with E-Selectin and P-Selectin

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Summary

We have compared the ability of human α/β and γ/δ T lymphocytes to adhere to selectin-bearing substrates, an interaction thought to be essential for homing and localization at sites of inflammation. Both T cell populations form rolling adhesions on E- and P-selectin substrates under physiologic flow conditions. Although equivalent to α/β T cells in binding to E-selectin, γ/δ T cells demonstrated greater ability to adhere to P-selectin that was purified or expressed on the surface of activated, adherent platelets. Under static conditions, 80% of γ/δ T cells and 53% of α/β T cells formed shear-resistant adhesions to P-selectin, whereas only 30% of γ/δ and α/β T cells adhered to E-selectin. The enhanced ability of γ/δ T cells to adhere to P-selectin cannot be attributed to differences in expression of the P-selectin glycoprotein ligand (PSGL-1), as all α/β T cells versus $\sim 75\%$ of γ/δ T cells expressed PSGL-1. Both cell populations expressed a similar percentage of the carbohydrate antigens sialyl Lewis^x and cutaneous lymphocyte-associated antigen. Depletion of lymphocyte populations or T cell clones bearing these oligosaccharides with the monoclonal antibody CSLEX-1 and HECA-452, respectively, resulted in a substantial reduction in adhesion to E-selectin and slight reduction in adhesion to P-selectin under flow conditions. Treatment of cells with an endopeptidase that selectively degrades O-sialomucins such as PSGL-1, abolished P-selectin but not E-selectin adhesion. Removal of terminal sialic acids with neuraminidase or protease treatment of cells abrogated cell adhesion to both selectin substrates. These results provide direct evidence for the presence of distinct E- and P-selectin ligands on T lymphocytes and suggest that γ/δ T cells may be preferentially recruited to inflammatory sites during the early stages of an immune response when P-selectin is upregulated.

Human T lymphocytes consist of two distinct cell populations that express different TCRs (1, 2). The majority of peripheral blood T cells and thymocytes express TCR- α/β . A smaller subset of cells bearing TCR- γ/δ constitute on average 5% of peripheral blood T lymphocytes and 0.2–0.9% of postnatal thymocytes (3, 4). Both populations of lymphocytes can be found throughout virtually all lymphoid organs, although γ/δ T cells are markedly enriched in the intestinal epithelium in mice and to a lesser degree in humans relative to other sites (5, 6). Although it has not been fully established whether γ/δ T cells function in a similar or distinct manner from α/β T cells during an immune response, these two populations of T lymphocytes possess several distinctive features. Whereas CD4 and CD8 expression phenotypically divides the α/β T cell population into distinct subsets that differ in their function (helper vs. cytotoxic) and MHC restriction (class II vs. class I), most γ/δ T cells lack expression of CD4 and

CD8 (5, 7, 8). Moreover, γ/δ T cells show no apparent restriction to conventional class I or class II MHC molecules, yet are capable of effector functions associated with α/β T cells such as cytotoxicity and secretion of multiple cytokines (9). Although both lymphocyte populations can be identified at sites of inflammation, γ/δ T cells are significantly enriched in various bacterial and parasitic infections, especially at particular stages of infection (10). γ/δ T cells also increase in certain plaque lesions in multiple sclerosis, in the intestinal epithelium in celiac disease, and in rheumatoid synovial tissue (11–15). In contrast, a subset of α/β T cells has been shown to predominate in chronic delayed-type hypersensitivity granulomas (16). Thus, α/β and γ/δ T cells may display preferential patterns of localization during specific inflammatory responses. These differences may result in part from distinct expression on the α/β and γ/δ T cell subsets of receptors for endothelial adhesion molecules that regulate lymphocyte traffic.

To participate in an immune response, circulating α/β and γ/δ T cells must first be recruited from the vasculature to sites of inflammation. Recent *in vivo* and *in vitro* studies have suggested a multistep process for neutrophil accumulation on activated endothelium that involves selectin-mediated tethering and rolling followed by activation-dependent, β_2 -integrin-mediated firm adhesion or "sticking." Although much remains to be learned about the traffic signals for T lymphocytes, evidence is accumulating for a similar multistep paradigm, with the modification that α_4 integrins can contribute to both the tethering and firm adhesion steps (17). Both P-selectin, which is upregulated from intracellular storage granules on thrombin-activated platelets and endothelium, and E-selectin, which is induced after longer time periods on cytokine-stimulated endothelial cells, have been shown to mediate Ca^{2+} -dependent adhesion of T cells (18–23). Interactions with these selectins have been shown to require sialylated oligosaccharide ligands that are closely related in structure to sialyl Lewis^x (sLe^x)¹ (24). An O-glycoprotease-sensitive sialomucin termed P-selectin glycoprotein ligand (PSGL) 1, which mediates P-selectin-dependent adhesion of myeloid cells, is also present on α/β T cells and may serve a similar function (23, 25, 26). PSGL-1 expression has not been examined on γ/δ T cells. E-selectin binding requires a distinct ligand that is endopeptidase insensitive (27–30). T cells expressing a carbohydrate antigen termed the cutaneous lymphocyte-associated antigen (CLA), as defined by reactivity with the mAb HECA-452, are the predominant E-selectin-binding population of lymphocytes (18, 20, 21). Twice as many γ/δ T cells as α/β T cells have been reported to express this carbohydrate epitope (20). Bovine γ/δ T cells demonstrate significant binding to both E- and P-selectin, yet do not express CLA (31, 32). In this study, we have compared human α/β and γ/δ T cells with respect to interactions with the vascular selectins, E- and P-selectin. We have examined the selectin ligands expressed on these two lymphocyte populations, evaluated the role of sLe^x/CLA in mediating E- and P-selectin adhesion, and provided a comparison of shear-dependent attachment and rolling interactions mediated by the vascular selectins. We find that both α/β and γ/δ T cells demonstrate preferential attachment and rolling on P-selectin as opposed to E-selectin, with γ/δ T cells showing the strongest interaction with P-selectin, and that distinct glycoprotein ligands are required for adhesion of T lymphocytes to E- and P-selectin.

Materials and Methods

Antibodies. Rb 3026, a rabbit polyclonal antibody to the fucosylated extracellular portion of PSGL-1 (33), was a gift of Drs. D. Cumming and R. Camphausen (Genetics Institute, Cam-

bridge, MA) and was used at a 1:100 dilution. The mAb G1 to P-selectin, IgG1 (34), and BB11 to E-selectin, IgG2b (35), were obtained from Dr. R. McEver (University of Oklahoma, Oklahoma City, OK) and Dr. R. Lobb (Biogen, Cambridge, MA), respectively. The biotinylated mAb BMA 031 (pan-TCR α/β , IgG1) and TCR- δ 1 (pan-TCR- γ/δ , IgG1) have been described (36). mAb to CLA (HECA-452, IgM) (37), sLe^x (CSLEX-1, IgM) (38), and sialyl Le^a (sLe^a) (2D3, IgM) (39), were obtained from the Fifth International Workshop on Human Leukocyte Differentiation Antigens. Nonbinding isotype-matched antibodies were used as negative controls. All antibodies, either dilutions of ascites (1:200) or purified IgG (10 $\mu\text{g}/\text{ml}$), were used at saturating conditions.

Cell Purification. PBMC and neutrophils were isolated by Ficoll-Hypaque gradient centrifugation (40). $\text{CD}3^+$ T lymphocytes were purified from PBMC by negative selection (41, 42). Purified α/β and γ/δ T cells were prepared from single-donor human platelet pheresis residues (which typically contained 3–10% γ/δ T cells) by positive selection using magnetic cell sorting according to the manufacturer's specifications (Miltenyi Biotec, Sunnyville, CA). Briefly, PBMC were resuspended to a concentration of 2×10^7 cells/ml in PBS, 1% BSA, 5 mM EDTA, pH 7.4 (PBE), and incubated with saturating concentrations (range 5–10 $\mu\text{g}/\text{ml}$) of either a biotinylated mAb BMA 031 or TCR- δ 1 for 30 min on ice. After washing twice in PBE, a 1:100 dilution of FITC-conjugated streptavidin (CALTAG Laboratories, San Francisco, CA) was added for 30 min on ice. The cells were washed in PBS, pH 7.4, incubated with magnetic microbeads conjugated to biotin (MACS; Miltenyi Biotec) for 10 min at 4°C, washed, and then passed over an AS1 magnetic column. Immunofluorescence flow cytometry revealed that the purified T lymphocyte preparations contained >95% α/β and γ/δ T cells. All T cell populations had >95% cell viability as determined by trypan blue exclusion. After isolation, the cells were stored in HBSS (Gibco Laboratories, Grand Island, NY) supplemented with 10 mM Hepes, 2 mM CaCl_2 , and 0.2% human serum albumin, pH 7.4 (assay medium and used within 6 h (T lymphocytes) or 4 h (neutrophils)).

For depletion of T cell populations expressing carbohydrate epitopes recognized by the mAb CSLEX-1 and HECA-452, negatively selected $\text{CD}3^+$ T cells were incubated with a 1:100 dilution of these mAbs for 30 min on ice. The cells were subsequently washed twice in PBE and incubated with rat anti-mouse IgM or mouse anti-rat κ magnetic microbeads (Miltenyi Biotec), respectively, for 30 min on ice and then passed over a magnetic column.

Derivation and Maintenance of γ/δ T Cell Clones. T cell clones were derived from purified γ/δ T lymphocytes either by limiting dilution or bead selection followed by PHA stimulation (9). The cells were maintained by periodic restimulation with allogeneic PBL and EBV-transformed B cell feeders and PHA-P.

Flow Cytometry. For one-color immunofluorescence, cells were incubated with 50 μl of antibody, washed twice, and resuspended in either 30 μl of FITC-conjugated goat F(ab')₂ anti-mouse Ig (Zymed Laboratories, Inc., South San Francisco, CA), or FITC-conjugated goat F(ab')₂ anti-rabbit Ig (Zymed Laboratories, Inc.) (43). For two-color flow cytometry, purified FITC-streptavidin-labeled α/β and γ/δ T cells were incubated with 20 $\mu\text{g}/\text{ml}$ human Ig (Sigma Chemical Co., St. Louis, MO) to block nonspecific interactions, incubated with 50 μl of antibody, washed twice, and then incubated with PE-conjugated goat F(ab')₂ anti-mouse IgM (Tago, Inc., Burlingame, CA) or PE-conjugated goat F(ab')₂ anti-rabbit IgG (CALTAG Laboratories) at the recommended dilutions. mAb 2D3 (anti-sLe^a, IgM) was used as a con-

¹ Abbreviations used in this paper: C, cytolytic; CLA, cutaneous lymphocyte-associated antigen; LFU, linear fluorescence unit; NC, noncytolytic; PBE, PBS plus BSA plus EDTA; PSGL, P-selectin glycoprotein ligand; sLe^x, sialyl Lewis^x; sLe^a, sialyl Lewis^a.

trol for mAb CSLEX-1 and HECA-452. PE conjugated Leu-8 (Becton Dickinson & Co., San Jose, CA) and PE-conjugated CD45R0 (Amac, Inc., Westbrook, ME) were used to detect surface expression of L-selectin and memory phenotype on FITC-labeled α/β and γ/δ T cells, respectively. PE-conjugated IgG2a (CALTAG Laboratories) was used as control. Samples were analyzed on a FACScan® flow cytometer (Becton Dickinson & Co.). Propidium iodide was used to gate out nonviable cells. Fluorescence intensity standards (CALTAG Laboratories) were run for each experiment to quantitate and compare surface expression of the selectin ligands among various populations of leukocytes.

Preparation of Selectin-containing Lipid Bilayers. Recombinant full-length human E-selectin was purified from a CHO cell line transfected with E-selectin cDNA provided by Dr. R. Lobb by immunoaffinity chromatography using anti-E-selectin mAb BB11 coupled to Sepharose (35). P-selectin, purified from human platelets, was a generous gift of Dr. R. McEver (44). Liposomes containing the reconstituted selectins were prepared by the method of octyl-glucoside (Sigma Chemical Co.) dialysis, and planar bilayers were formed by incubating an aliquot of liposome suspension on glass slides (45). Densities of E-selectin and P-selectin were adjusted to give equal binding of HL-60 cells and neutrophils. Site densities for E- and P-selectin-containing bilayers were 200 sites and 400 sites/ μm^2 , respectively, as estimated from previous studies based on neutrophil detachment profiles and rolling velocities (45, 46).

Platelet Substrate. Platelet monolayers were formed on 3-aminopropyltriethoxysilane-treated slides and activated with thrombin as described (43).

Laminar Flow Assays. A glass slide containing either adherent platelets or selectin proteins incorporated into lipid bilayers was assembled in a parallel-plate laminar flow chamber (260- μm gap thickness) in which a uniform wall shear stress is generated (45). The flow chamber was mounted on the stage of an inverted phase contrast microscope (Diaphot-TMD; Nikon Inc., Garden City, NJ). For detachment, tethering, and rolling assays, data on each cell population and treatment within an experiment was obtained from the identical field of view (0.67 mm^2). HBSS containing 5 mM EDTA was infused between each data set, and all cells were released, confirming specific interactions. Rarely, data were discarded because not all cells were released.

For detachment assays, T cells ($2 \times 10^6/\text{ml}$) were injected through a side port and allowed to settle for 5 min. Controlled flow was applied and doubled at 20-s intervals from 0.73 to 36 dyn/cm^2 . The number of cells remaining bound at each shear stress was compared.

For tethering assays, T cells, neutrophils, or HL-60 cells ($10^6/\text{ml}$) were perfused over selectin substrates for 3 min at the desired shear stress, and the number of cells that tethered in the field of view was measured. A tether was defined as a cell bound to the substrate for ≥ 5 s.

Rolling velocities were determined for a minimum of 40 cells per field of view in two experiments performed on different days. Cells were allowed to settle under static conditions, and flow was increased incrementally from 0.73 to 36 dyn/cm^2 . Velocities on the selectin bilayers were comparable whether cells were bound during shear flow or at stasis and then subjected to shear flow.

All experiments were recorded and results quantitated by analysis of images videotaped with a video camera (model TEC-470 CCD; Optronics Engineering, Goleta, CA) and recorder (model Hi 8 CVD-1000, Sony). For antibody inhibition studies, platelets or the selectin bilayers were incubated with 20 $\mu\text{g}/\text{ml}$ of mAb for 30 min at room temperature before the detachment assay.

Cell Treatments. To remove terminal cell surface sialic acids, peripheral blood $\text{CD}3^+$ T lymphocytes were incubated with 0.1 U/ml *Vibrio cholera* neuraminidase (Calbiochem Corp., San Diego, CA) for 1 h at 37°C in assay medium. To remove mucinlike domains from cell surface proteins, lymphocytes were incubated with 50 $\mu\text{g}/\text{ml}$ of *Pasteurella haemolytica* O-glycoprotease (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada) for 1 h at 37°C in assay medium. Control cells were incubated under similar conditions in the absence of the enzyme. Cells were washed twice in assay medium and stored at 4°C after completion of enzymatic treatments.

Statistical Analysis. The Wilcoxon rank sum test (nonparametric) was used for data comparison because of a lack of normal distribution.

Results

Adhesion of Unstimulated Neutrophils and T Lymphocytes to Surface-adherent Platelets. Activated, adherent platelets supported tethering in flow of neutrophils and T lymphocytes within the identical range of shear stresses (up to 3.6 dyn/cm^2) (Fig. 1). Neutrophils, however, were superior to T lymphocytes with respect to the number of cells bound at every wall shear stress tested (2–3.5-fold higher). This difference was also apparent in static assays where only 60% of T lymphocytes versus 100% of neutrophils bound to platelets (data not shown). Incubation of adherent platelets with the anti-P-selectin mAb G1 (20 $\mu\text{g}/\text{ml}$) completely abolished adhesion under flow and static conditions (Fig. 1), showing tethering was specifically mediated by P-selectin.

To determine whether α/β or γ/δ T lymphocytes demonstrated preferential binding to the immobilized platelet substrate, the phenotype of bound cells was ascertained by immunofluorescence labeling with a pan- γ/δ TCR mAb. Surprisingly, $\sim 35\%$ of T cells attached under flow conditions (0.73 dyn/cm^2) were TCR- γ/δ^+ . By contrast, only 5% of the initial population of purified T cells were TCR-

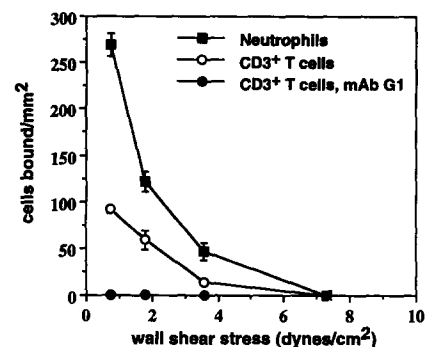


Figure 1. $\text{CD}3^+$ T lymphocytes and neutrophils tether to surface-adherent platelets under physiologic flow conditions. Platelets were allowed to adhere to aminopropyltriethoxysilane-coated slides to form a monolayer and activated with thrombin. Slides were incorporated in a parallel wall flow chamber. Adherence of T lymphocytes or neutrophils after 3 min at the indicated wall shear stress was quantitated. mAb G1 was used to pretreat adherent platelets for 15 min and was continually present during the assay. Error bars represent the SD of three different sets of experiments performed in duplicate.

γ/δ^+ (results not shown). All T cells formed rolling adhesions, and attachment was completely abolished in the presence of the anti-P-selectin mAb G1 or 5 mM EDTA.

Adhesion of α/β and γ/δ T Lymphocytes to Artificial Planar Bilayers Containing Purified E- or P-Selectin. To directly compare selectin-mediated attachment of α/β versus γ/δ T cells under physiologic flow conditions, purified populations of lymphocytes were perfused for 3 min over lipid bilayers containing either E- or P-selectin. Selectins were incorporated into artificial bilayers at concentrations that yielded a similar number of attachments of the promyelocytic cell line HL-60 at a shear stress of 0.73 dyn/cm² (112 ± 14 vs. 114 ± 19 cells/mm², respectively). Tethering of γ/δ T cells to purified P-selectin was twofold greater than for the α/β population ($P < 0.001$) at each of the two wall shear stresses tested (Fig. 2 A). By contrast, binding to E-selectin bilayers was not significantly different for the two T cell populations ($P = 0.12$) (Fig. 2 B). On all substrates, cells rolled subsequent to tethering. Unlike HL-60 cells, which bound identically to P-selectin and E-selectin, both α/β and γ/δ T cells demonstrated preferential binding to P-selectin compared with E-selectin. At a shear stress of 0.73 dyn/cm², α/β and γ/δ T cells bound 1.4- and 3-fold, respectively, better to P-selectin than E-selectin ($P < 0.001$). Interestingly, increasing the wall shear stress 2.5-fold to 1.8 dyn/cm² eliminated tethering of α/β and γ/δ T cells to E-selectin (Fig. 2 B), but only resulted in a slight drop in adherence to P-selectin (Fig. 2 A). Incubation of E- or P-selectin-containing bilayers with the anti-E-selectin

mAb BB11 or anti-P-selectin mAb G1, respectively, completely inhibited attachment at all flows (results not shown).

Comparison of Functional Ligands and Resistance to Shear-induced Detachment Forces. The percentage of α/β and γ/δ lymphocytes that expressed functional cell surface ligand(s) for E- and P-selectin was examined under static conditions. T cells were allowed to settle onto the artificial planar membranes, and after 5 min of contact, controlled flow at a wall shear stress of 0.73 dyn/cm² was used to create a detachment force. A significantly higher percentage of γ/δ T cells (80%) than α/β T cells (53%) ($P < 0.001$) adhered to P-selectin bilayers (Fig. 3). All cells formed rolling adhesions under these conditions. Furthermore, a lower (30%) and equivalent percentage of each cell type ($P = 0.52$) bound to bilayers containing E-selectin. These results correlate with preferential tethering in flow of γ/δ T cells to P-selectin and lower tethering to E-selectin that is comparable for α/β and γ/δ T cell subsets. HL-60 cells bound equivalently to either selectin substrate under static conditions ($>95\%$; data not shown).

Although a greater percentage of γ/δ than α/β T cells tethered to P-selectin in flow, both populations of lymphocytes demonstrated similar resistance to shear-induced detachment forces (Fig. 4 A) and rolled at similar velocities on P-selectin (Fig. 5 A). This suggests that an equivalent amount of adhesive interactions are formed between P-selectin and its ligand expressed on either cell type. In contrast, α/β T cells were more shear resistant than γ/δ T cells on E-selectin (Fig. 4 B), especially at the higher wall shear stresses ($P = 0.08$ at 7.3 dyn/cm² and $P = 0.05$ at 36 dyn/cm²). The α/β T cells rolled more slowly than the γ/δ T cells on E-selectin at higher shear stress (Fig. 5 B), correlating with greater resistance to detachment. The α/β T cells had similar resistance to detachment and rolling velocities on E-selectin and P-selectin; thus, inability of α/β T cells to accumulate on E-selectin at 1.8 dyn/cm² (Fig. 2 B) reflects a deficiency in tethering rather than in subsequent rolling interactions.

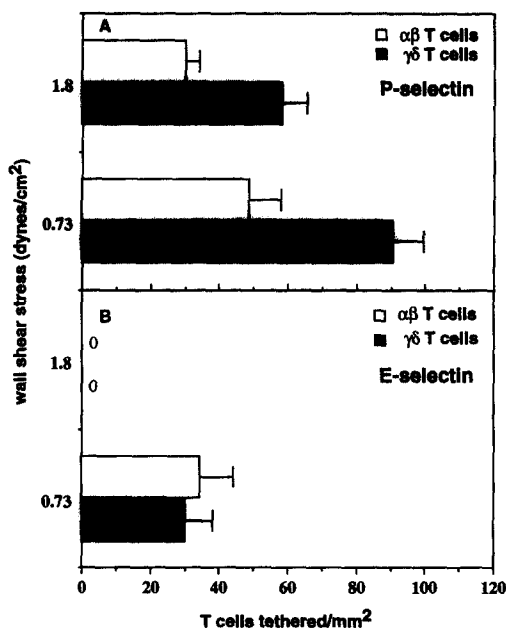


Figure 2. Tethering of α/β and γ/δ T lymphocytes to selectin-containing bilayers in flow. T cells were infused through a parallel-plate flow chamber containing purified P-selectin (A) or E-selectin (B) in phosphatidylcholine bilayers. Tethering was quantitated during 3 min of continuous flow. Bars show the SD of three different sets of experiments performed in duplicate.

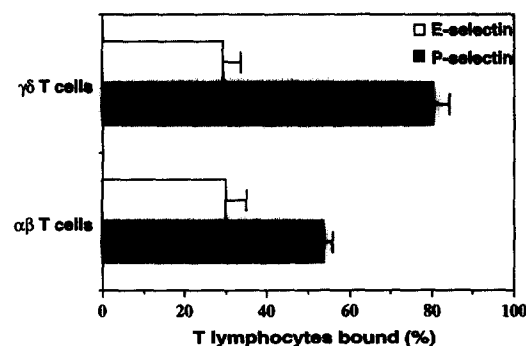


Figure 3. The percentage of α/β and γ/δ T lymphocytes expressing functional ligand(s) for P- or E-selectin determined by static adhesion followed by washing in flow. T cells were allowed to settle onto bilayers containing P- or E-selectin. After 5 min of contact, a shear stress of 0.73 dyn/cm² was applied for 10 s, and the percentage of cells that remained adherent was determined. Error bars represent the SD of three independent experiments performed in duplicate.

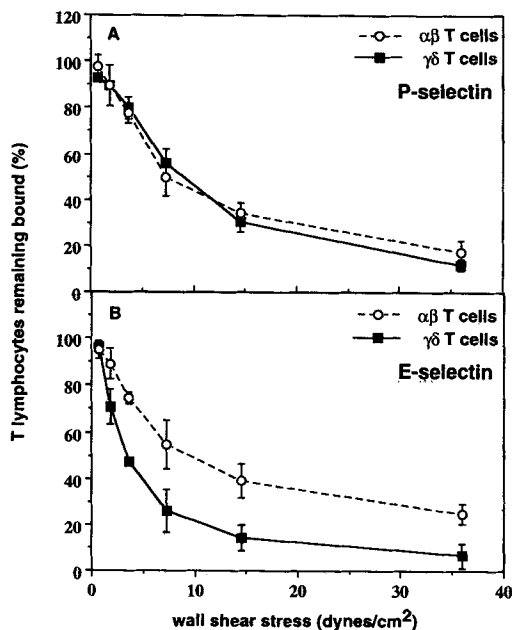


Figure 4. Strength of rolling adhesions of α/β and γ/δ T lymphocytes on selectins in lipid bilayers. T cells that settled onto bilayers containing P-selectin (A) or E-selectin (B) as described in Fig. 3 were subjected to staged increments of shear. Cells remaining bound after 20 s at each shear stress are expressed as the percentage of T lymphocytes that were bound after the initial period at 0.73 dyn/cm². Error bars represent the SD of three independent experiments performed in duplicate.

Expression of Antigens Associated with Selectin Function of α/β and γ/δ T Cells. Two-color flow cytometry was used to define patterns of expression of PSGL-1, sLe^x, CLA (HECA-452 antigen), and L-selectin on purified populations of T

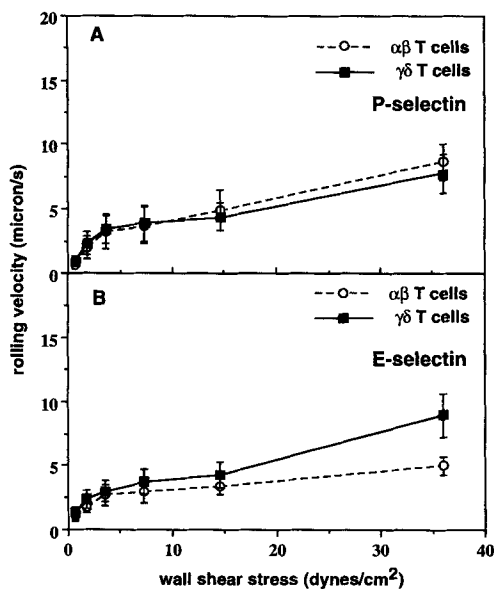


Figure 5. Rolling velocities of α/β and γ/δ T cells. Cells were allowed to adhere to bilayers containing P-selectin (A) or E-selectin (B) and subjected to incremental increases in shear stress as described in Figs. 3 and 4. Rolling velocities were determined for 40–50 cells in two independent experiments. Each point represents the mean \pm SD.

cells (Fig. 6). In five different donors, PSGL-1 was present on essentially all α/β T cells ($96 \pm 3\%$) and on a subset of γ/δ T cells ($74 \pm 6\%$). The percentage of γ/δ T cells expressing the PSGL-1 sialomucin correlated with the percentage of γ/δ T cells bound to P-selectin under static conditions. The lack of a similar correlation for α/β T cells suggests that a significant proportion of these cells do not bear the appropriate carbohydrate structures on ligand(s) such as PSGL-1 necessary for adhesion to P-selectin. A similar percentage of α/β and γ/δ T cells expressed the carbohydrate antigens sLe^x ($42 \pm 4\%$ and $37 \pm 10\%$, respectively) and HECA-452 antigen (mean of $41 \pm 15\%$ and $40 \pm 8\%$, respectively). This correlated with the similar binding profiles

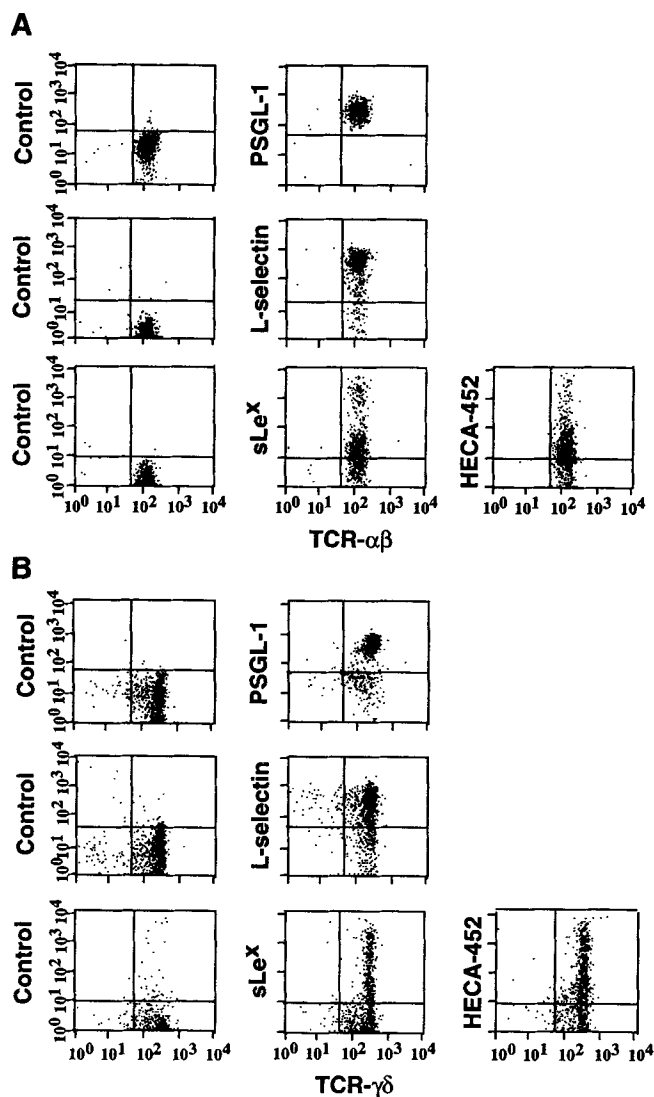


Figure 6. Expression of antigens on T lymphocytes associated with selectin function. T cells from the same individual, positively selected for expression of α/β TCR (A) or γ/δ TCR (B), were stained with antibodies to the various cell surface molecules and analyzed by two-color flow cytometry (5,000 events shown). The quadrant markers were drawn based on appropriate negative controls. Overall identical results were obtained using cells from five different blood donors. Staining with FITC and PE is shown on the abscissa and ordinate, respectively.

of γ/δ and α/β T cells under flow and static conditions on E-selectin. L-selectin was present on $87 \pm 3\%$ of α/β versus $71 \pm 5\%$ of γ/δ T cells, and incubation of each lymphocyte population with the anti-L-selectin mAb Dreg 56 did not alter adhesion to either selectin bilayer under flow conditions (results not shown).

Strength of Adhesion of γ/δ T Cell Clones to E-Selectin, but Not P-Selectin, Correlates with sLe^x Surface Expression. To examine the importance of sLe^x in interactions with the vascular selectins, γ/δ T cell clones with heterogeneous expression of the sLe^x carbohydrate antigen were used in detachment assays. These clones derived from peripheral blood were of the V γ 2 V δ 2 subset, and varied both in CD4 and CD8 expression and cytolytic (C) and noncytolytic (NC) function (Fig. 7 A). The level of expression of sLe^x as shown by linear fluorescence units (LFU) varied >100-fold. For comparison, PSGL-1 was expressed on $\geq 94\%$ of the five clones studied with an LFU that varied only fivefold. All clones were 100% CD45R0⁺ and did not express L-selectin (data not shown). Four clones, HF2, CP.1.5, HD.108, and CP.1.15, demonstrated preferential binding to P-selectin as opposed to E-selectin (Fig. 7 B) and similar resistance to shear on P-selectin (Fig. 7 C). There was heterogeneity among these four clones in the

percentage of cells that remained adherent in increasing shear to E-selectin (Fig. 7 C). In contrast, JN23, the clone with the highest level of sLe^x expression, demonstrated equivalently high adhesion (Fig. 7 B) and resistance to shear stress-induced detachment forces (Fig. 7 C) on E- and P-selectin-containing bilayers. Assays of tethering in flow at 0.73 dyn/cm² showed that JN23 tethering during 3 min on bilayers containing E-selectin and P-selectin was 92 ± 14 and 86 ± 14 cells/mm², respectively, an efficiency similar to HL-60 cells (see above). There was a correlation between expression of sLe^x and strength of adhesion to E-selectin, with a correlation coefficient of 0.87 (Fig. 7 D). By contrast, there was little correlation between sLe^x expression and strength of adhesion to P-selectin, with a correlation coefficient of 0.36 (Fig. 7 D). Only the clone with the highest sLe^x expression showed stronger adhesion to P-selectin, whereas the other four clones varied widely in sLe^x expression, yet showed similar adhesion under static conditions and resistance to shear. Similar results were obtained for the carbohydrate epitope(s) recognized by the mAb HECA-452 (data not shown). PSGL-1 surface expression did not correlate with adhesion to either E-selectin or P-selectin (correlation coefficients of 0.05 and 0.35, respectively).

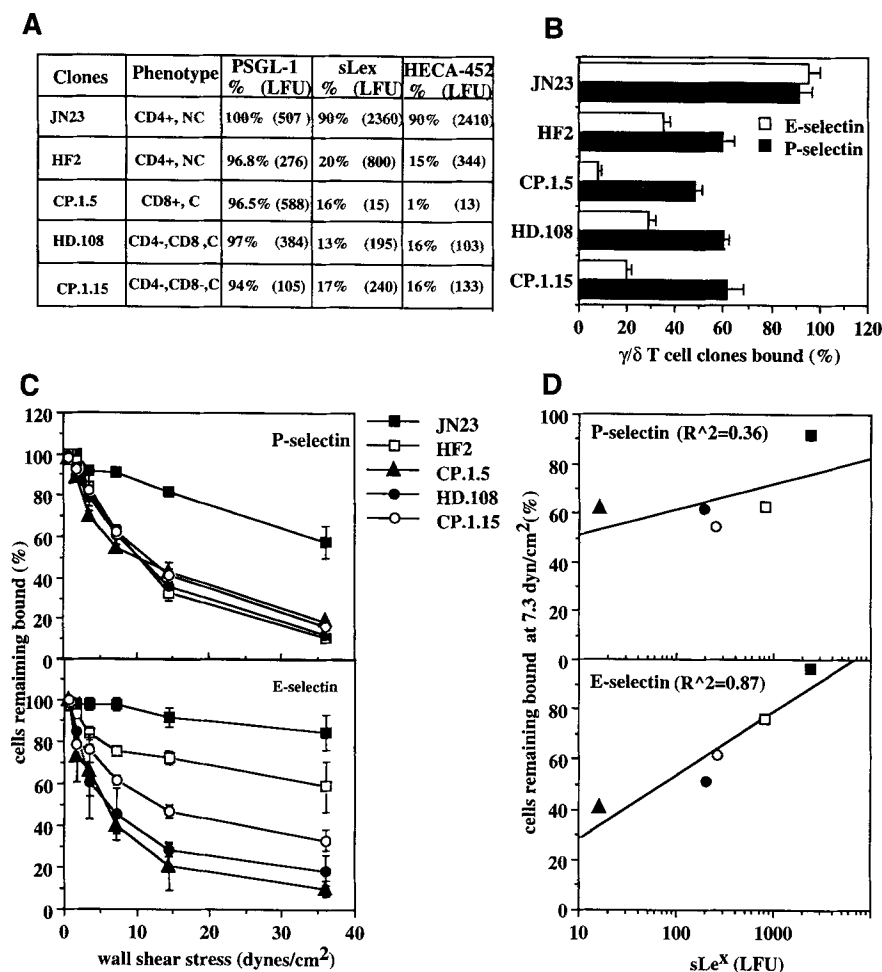


Figure 7. Phenotype and resistance to shear stress-induced detachment of γ/δ T cell clones on bilayers containing E-selectin and P-selectin. (A) Comparison of PSGL-1, sLe^x (CSLEX-1), and HECA-452 (CLA) expression on γ/δ T cell clones as determined by one-color immunofluorescence flow cytometry. Phenotypes are represented as either cytolytic (C) or noncytolytic (NC). Surface expression is shown both as percentage of positive cells (%) and LFU of positive cells. (B–D) γ/δ T cell clones were allowed to settle onto bilayers containing P- or E-selectin, and after 5 min, shear stress was applied in staged increments as described in Figs. 3 and 4. Error bars denote the SD of triplicates in one representative experiment. (B) Percentage of the cells that settled on selectin bilayers and remained bound after 10 s of flow at 0.73 dyn/cm². (C) The percentage of cells adherent at 0.73 dyn/cm² that remained adherent and rolled at increasing wall shear stress. (D) Correlation of sLe^x expression on clones and resistance to detachment at a shear stress of 7.3 dyn/cm².

The Effects of CSLEX-1 and HECA-452 Antigen Depletion on Peripheral Blood T Cell Attachment to E- and P-Selectin Bilayers under Flow Conditions. Immunomagnetic depletion with CSLEX-1 mAb of γ/δ clone HF.2 resulted in complete removal of cells expressing CSLEX-1 and HECA-452 epitopes. This treatment reduced tethering of HF.2 δ cells on E-selectin from 39 ± 4 to 0 cells/mm²; by contrast, accumulation on P-selectin of 53 ± 8 cells/mm² was changed by depletion to 31 ± 7 cells/mm². Similar experiments were carried out on purified peripheral blood CD3⁺ T lymphocytes; pu-

rified α/β and γ/δ T cell subsets could not be used for these experiments because of prior positive magnetic selection. Depletion with CSLEX-1 mAb removed 97% of CSLEX-1⁺ cells and 80% of HECA-452⁺ cells (Fig. 8 A), and reduced accumulation in flow on E-selectin by 73% ($P < 0.001$) (Fig. 8 B). In contrast, accumulation of P-selectin was reduced by 38% (Fig. 8 B). Depletion with HECA-452 mAb removed >95% of CSLEX-1⁺ and HECA-452⁺ cells (Fig. 8 A), and reduced accumulation of CD3⁺ lymphocytes by 93% on E-selectin (Fig. 8 B) but only 32% on P-selectin (Fig. 8 B).

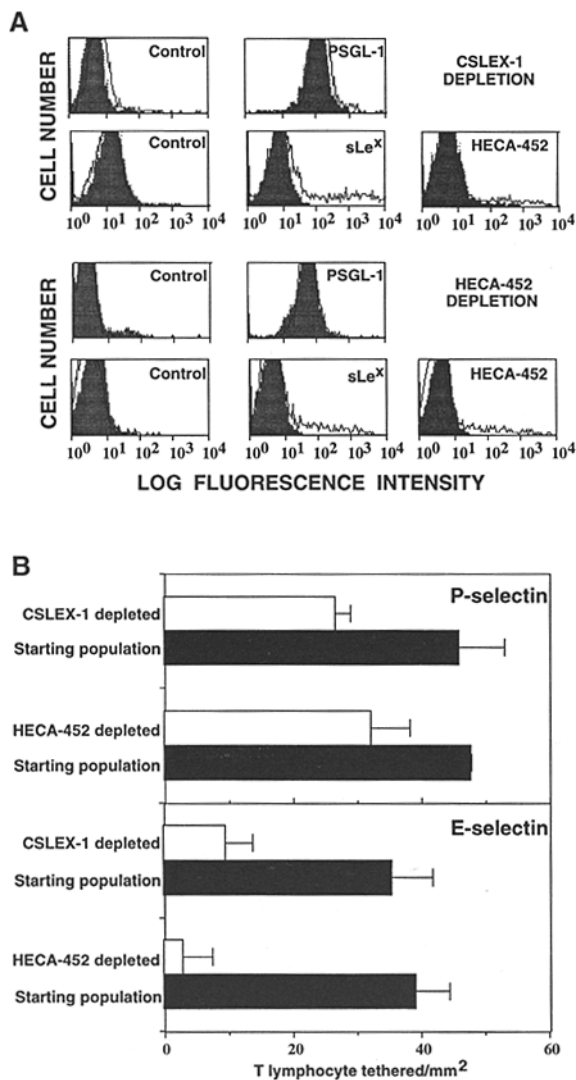


Figure 8. Depletion of T cell populations expressing carbohydrate epitopes and effect on binding to P-selectin and E-selectin. (A) Peripheral blood T lymphocytes were depleted with either CSLEX-1 mAb (upper panel) or HECA-452 mAb (lower panel) by negative immunomagnetic selection. Nondepleted (open bars) and depleted (shaded bars) populations of T cells were stained with the indicated mAb and FITC anti-Ig and subjected to flow cytometry. (B) CD3⁺ T lymphocytes or fractions depleted with CSLEX-1 or HECA-452 mAb were infused over bilayers containing P-selectin (upper panel) or E-selectin (lower panel) at a shear stress of 0.73 dyn/cm² for 3 min and cell tethering was determined. Error bars represent the SD of two different sets of experiments perfused in duplicate.

Discussion

The starting point for these studies was the observation that T lymphocytes accumulated and rolled on monolayers of activated platelets with an efficiency only twofold lower than neutrophils, and in the same range of shear stresses, up to 3.6 dyn/cm². Furthermore, we found that γ/δ T cells preferentially accumulated on platelet monolayers, raising the question of whether γ/δ and α/β T cells might display differences in interactions with selectins that contribute to selectivity in emigration from the bloodstream. We find that γ/δ T cells are more efficient than α/β T cells in binding to P-selectin in flow, and also contain a higher proportion of cells that bear a functional P-selectin ligand, as shown by binding in static conditions. A lower proportion of both α/β and γ/δ T cells, 30%, bear functional ligands for E-selectin. Furthermore, tethering to P-selectin was more robust; whereas T cells tethered to both E-selectin and P-selectin at 0.73 dyn/cm², T cells tethered only to P-selectin at 1.8 dyn/cm². We further characterized heterogeneity among γ/δ T cell clones and among CD3⁺ blood T lymphocytes in expression of ligands for E-selectin and P-selectin, and showed that sLe^x and CLA are good markers for T cells that bind to E-selectin, but not for cells that bind to P-selectin.

The preferential binding of T lymphocytes, and of γ/δ cells, to P-selectin compared with E-selectin has not been previously reported. We have found that 60% of purified T cells bind to platelet monolayers, and that 80% of γ/δ and 54% of α/β T cells bind to artificial bilayers containing P-selectin. Previous studies have reported lower values, which may reflect assays that are less sensitive. For instance, a mean of 27% of CD3⁺, 22% of CD4⁺, and 36% of CD8⁺ T cells were found to bind in suspension to P-selectin on activated platelets as determined by flow cytometry (19). In a similar assay, a mean of $6.0 \pm 5\%$ of CD4⁺ T cells and $16.6 \pm 8.2\%$ CD8⁺ T cells were capable of interacting with activated platelets (22). In another report, only chronically stimulated, not resting, CD4⁺ T cells bound to P-selectin chimera insoluble as examined by indirect immunofluorescence (47). In contrast to human T cells, a significant proportion of bovine γ/δ T cells were shown to tether equally well to P-selectin and E-selectin, whereas the α/β population in this species tethered minimally to either substrate (31). Our results suggest that human T lymphocyte interac-

tions with P-selectin may be more important in lymphocyte extravasation than previously thought. Furthermore, it is tempting to speculate that this interaction may also be involved in T lymphocyte homing to skin, as previously described for E-selectin at cutaneous sites of chronic inflammation (30). This is supported by *in vivo* observations that P-selectin, but not E- or L-selectin, mediates spontaneous rolling of leukocytes in venules of murine skin in the absence of an inflammatory challenge (48). P-selectin may be involved in primary immune surveillance by virtue of its expression in anatomical regions where pathogens may preferentially invade. In this hypothetical scenario, P-selectin would not only mediate T cell recruitment during an acute inflammatory event where its expression is transiently induced, but also function as a homing receptor for a subset of immune-competent lymphocytes that recognize frequently encountered epithelial antigens. This interaction may also contribute to increased γ/δ -to- α/β T cell ratios in epithelium compared with blood or lymphatic organs. The higher proportion of γ/δ than α/β T cells that interacted with P-selectin appeared to reflect a difference in the percentage of cells that expressed a functional P-selectin ligand rather than a difference in the amount of ligand expressed on the γ/δ and α/β T cell subsets, because once cells attached, resistance to detachment and rolling velocities were similar for both T cell subsets.

Glycoproteins decorated with HECA-452 epitope (CLA antigen) and/or sLe^x have been proposed as the major ligand(s) on T cells for E-selectin (18, 49, 50). We found that the percentages of α/β and γ/δ T cells that expressed these oligosaccharides were equivalent to the percentages of these cells that bound to E-selectin. Furthermore, we observed that depletion of the HECA-452⁺ subsets or the CSLEX-1⁺ subsets within peripheral blood T lymphocytes and γ/δ T cell clones almost completely abolished or substantially reduced tethering to E-selectin (50). In contrast, more γ/δ and α/β T cells bound to P-selectin under static conditions than expressed these carbohydrate epitopes, suggesting a role for additional oligosaccharides. Further evidence supporting the requirement for distinct glycosylation or other posttranslational modifications of E-selectin and P-selectin ligands was provided by analysis of detachment profiles of T lymphocyte clones. Representative γ/δ T cell clones expressing various amounts of sLe^x and HECA-452 were observed to have similar binding and detachment profiles on P-selectin, but differed significantly with respect to shear resistance on E-selectin. We would expect comparable results on either selectin substrate if identical oligosaccharides and/or proteins were required for adhesion. Similarly, removal of T cell populations expressing sLe^x and/or HECA-452 antigen from peripheral blood T lymphocytes or γ/δ clones minimally reduced adhesion to P-selectin under flow conditions. We cannot rule out a role for sLe^x in P-selectin binding because a 30–40% reduction in P-selectin binding of peripheral blood T cells was noted in depletion studies. This may reflect heterogeneous glycosylation of the P-selectin ligand(s) expressed on these cells.

Previous studies have identified PSGL-1, a homodimeric

sialomucin from myeloid cells as a P-selectin ligand (33, 44, 51). Anti-PSGL-1 antibodies have been shown to completely inhibit binding of purified P-selectin to neutrophils as well as to peripheral blood T lymphocytes (25), and to inhibit adhesion of T cells to P-selectin (23). We found that essentially all α/β T cells express PSGL-1, as found previously for CD3⁺ T cells (23, 25). Thus, the finding that only 54% of α/β T cells bound to P-selectin suggests differential posttranslational modification of PSGL-1 among the α/β T cell subset. Further evidence is provided by studies involving T lymphocytes from cord blood. Although >95% of neonatal T cells express PSGL-1, <5% bind to P-selectin under static conditions (Diacovo, T. G., unpublished observation). Thus, it appears that expression of functional PSGL-1 may be induced during antigen-mediated naive virgin-to-memory T cell conversion in secondary lymphoid organs.

In contrast to α/β T cells, we found that $74 \pm 6\%$ ($n = 5$) of peripheral blood γ/δ T cells expressed PSGL-1 and that a similar percentage of cells, $80 \pm 4\%$ ($n = 3$), bound to P-selectin. We hypothesize that these two populations of T cells are identical. Whether PSGL-1 is selectively expressed on specific subsets of γ/δ T cells, such as the V δ 1 or V δ 2 population, requires further evaluation. We also found that digestion with O-glycoprotease of mucinlike molecules on purified peripheral blood T cells and γ/δ T cells abolished interactions with P-selectin but not E-selectin (data not shown), in agreement with previous studies on the importance of PSGL-1 on T cells for binding to P-selectin (23, 25, 52). Neuraminidase treatment of T cells abolished binding to both E-selectin and P-selectin (data not shown).

In summary, we have demonstrated that both human α/β and γ/δ T cells can interact with E-selectin and P-selectin under physiologic flow conditions and that they possess similar selectin ligands. Furthermore, sLe^x/HECA-452 antigen expression on both populations of lymphocytes correlates with E-selectin-dependent binding, whereas adhesion of T cells to P-selectin requires a sialomucin glycoprotein ligand bearing a unique carbohydrate structure. Although identical in these respects, the enhanced ability of γ/δ T cells to bind to P-selectin, an adhesion molecule associated with immediate inflammatory events, suggests that these cells may play a significant role in the early response to bacterial infections. This subset of T lymphocytes is well suited for immune surveillance because γ/δ T cells have been shown to recognize nonpeptide bacterial antigens presented through a novel extracellular pathway that does not require antigen internalization, processing, or expression of class I, class II, or CD1a, 1b, and 1c molecules (53, 54). Similar observations have not been reported for α/β T cells to date. Furthermore, γ/δ T cells have also been implicated in the pathogenesis and maintenance of rheumatoid arthritis, because they have been shown to accumulate in affected joints of these patients (15). Interestingly, P-selectin, derived from platelets and endothelium, has been shown to be the predominant adhesion molecule detected on the microvasculature in rheumatoid arthritis and is believed to

contribute to lymphocytic recruitment (55). Thus, γ/δ T cells may have a selective advantage to respond to specific disease processes by virtue of their preferential ability to ad-

here to P-selectin and thus may play a crucial role in providing primary host defense against invading organisms and in the maintenance of chronic inflammation.

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