Title
P-selectin glycoprotein ligand-1 mediated adhesion to P- and E-selectin under flow

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P-selectin Glycoprotein Ligand-1 Mediated Adhesion to P- and E-selectin Under Flow

by

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Abstract

We endeavored to determine whether P-selectin glycoprotein ligand-1 (PSGL-1), a leukocyte counter receptor implicated in accumulation of flowing neutrophils on P- and E-selectin, is sufficient to mediate attachment to and rolling on P- and E-selectin under flow and if so, to characterize the regions of PSGL-1 that are necessary and/or sufficient for these interactions. Previous reports have implicated the N-terminal anionic region of PSGL-1 in static adhesion to P- and E-selectin. Thus, we developed an in vitro model in which intact or mutated regions of the PSGL-1 N-terminus were coupled to 10 μm diameter polystyrene microspheres. In a parallel plate chamber with flow conditions mimicking those of post-capillary venules, live time videomicroscopy revealed that microspheres coated with the N-terminal 148 amino acids of PSGL-1 attached to and rolled on 4 hour TNF-α activated human umbilical vein endothelial cell (HUVEC) monolayers, which express E-selectin at high levels, and on Chinese Hamster Ovary monolayers stably expressing E- (CHO-E) or P-selectin (CHO-P). Treatment of these PSGL-1 microspheres with O-sialoglycoprotein endopeptidase, an enzyme that cleaves PSGL-1, abolished attachment to CHO-E and CHO-P monolayers. Microspheres coated with the amino-terminal 19 amino acids of PSGL-1 attached and rolled on both P- and E-selectin; however, a mutated form of PSGL-1 that lacks the anionic sequence at positions 5-11 attached and rolled on E- but not P-selectin. In addition, neuraminidase treatment greatly reduced attachment of microspheres coated with the 19 N-terminal residues to P- and E-selectin. Thus, as few as the 19 N-terminal amino acids of PSGL-1 are sufficient for attachment to and rolling on P-selectin and E-selectin, the N-terminal anionic polypeptide sequence is necessary for attachment to P- but not E-selectin, and sialylated moieties are necessary for attachment to P- and E-selectin. In addition, further studies suggest that PSGL-1 has more than one binding site sufficient for attachment to and rolling on E-selectin: a sLe^x-containing glycan coupled to a threonine at position 16 and one, or more, site(s) located between residues 20 and 148.

Introduction

The inflammatory response consists of multiple strictly regulated components which together make up a delicately controlled process with the potential for rapid amplification or dampening. The efficiency of this process depends greatly on one such component, white blood cell (leukocyte) recruitment. Leukocyte migration to a localized area of bacterial infection or tissue injury is a highly regulated journey that begins with the adhesion of a leukocyte to the vascular endothelium.

For a leukocyte to extravasate form the vascular compartment during the inflammatory response, it must undergo a multi-step adhesion cascade with the endothelium (Figure 1) (1-4). The cascade begins with leukocyte attachment or tethering (Step I) to and rolling (Step II) along the surface of the endothelium. These initial steps of the cascade are reversible. The leukocyte can then either detach from the endothelial surface to the flowing blood or undergo firm arrest (Step III), spreading (Step IV) and finally, transmigration (Step V) through the endothelium.

Leukocyte initial attachment to and rolling on vascular endothelium are crucial steps in regulation of leukocyte migration. A family of glycoproteins, known as selectins, have been shown to be involved in these initial steps of the adhesion cascade (1-9). The three known members of the selectin family, P-, E- and L-selectin, share a common structural motif: a N-terminal lectin-like binding domain, an epidermal growth factor-like region and a variable number of short consensus repeats similar to those found in complement-regulatory proteins (Figure 2) (7-9). P-selectin is constitutively synthesized in platelets and endothelial cells (Figure 3) and immediately stored in alpha granules and Weibel-Palade bodies, respectively (10, 11). Various mediators, including thrombin or histamine, or

secretogogues, such as A23187, can stimulate rapid mobilization of P-selectin to the cell surface (Figure 3). In contrast, E-selectin's expression is limited to activated endothelial cells (12). Tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) or certain Gram negative bacterial endotoxins induce E-selectin expression on cultured vascular endothelium with maximal surface expression 4 - 6 hours after exposure (Figure 3) and a subsequent decline to basal levels by 24 - 48 hours (13). L-selectin is constitutively expressed on the surface of neutrophils, monocytes and most lymphocytes. Many in vivo studies of tissues prepared for intravital microscopy and in vitro studies performed under flow have implicated L-selectin in leukocyte accumulation on activated endothelium (2, 5, 9, 14, 15). In addition, L-selectin has been shown to be an important molecule for lymphocyte recirculation in peripheral lymph nodes (8, 16).

P-selectin glycoprotein ligand-1 (PSGL-1), expressed as a homodimer of disulfide-linked subunits on the surface of neutrophils, monocytes, some lymphocytes and human promyelocytic HL60 cells has been implicated in promoting leukocyte adhesion to E- and P-selectin (17-23). PSGL-1 is a sialomucin that displays numerous, clustered O-linked glycans (24). Many of these glycans carry sialylated and fucosylated carbohydrates such as the tetrasaccharide sialyl-Lewis^x (sLe^x). Similar to L-selectin (25), PSGL-1 is localized on the tips of neutrophil microvilli (20); however, its localization on other leukocyte cell types has yet to be established. It has been postulated that the peripheral location of PSGL-1 on neutrophils is conducive for mediating the initial interaction between a flowing neutrophil and the vascular endothelium (20, 25).

The great majority of adhesion studies have been performed under static conditions. These studies utilize a variety of methods. One common method consists of radioactively labeling a cell or adhesion molecule and adding it to a well to which another molecule or cell has previously been fixed. After a given incubation time, the well is washed and

subsequently, the radioactivity or adhesion is determined. Although adhesion under static conditions provides valuable preliminary information, it is more relevant to study adhesion under conditions of fluid flow which mimic in vivo vascular fluid dynamics (26). Under flow the time that a ligand and its counter receptor are in the necessary proximity (26) and proper configuration for a potential adhesive interaction and the forces experienced by the relevant molecules more closely approximate those parameters in vivo.

Adhesion under flow has been studied in vivo with intravital microscopy and in vitro with videomicroscopy in conjunction with flow chambers that model blood flow in vivo. In general, adhesion with flow chambers is studied by perfusing cells over a substrate expressing adhesion molecules. The seminal in vitro studies of PSGL-1 adhesion under flow have relied on a single method (20, 27). After a given time of flowing cells over a substrate (on the order of minutes), the number of rolling cells on a defined area of the surface are determined, and this number has been interpreted to grossly correlate with the rate of cell attachment.

However, this interpretation is potentially flawed. The total number of cells on a surface can be expressed by the following equation:

(i) Accumulation Cells = Arrested Cells + Rolling Cells.

And a mass balance over a given area of the surface, yields:

(ii) Accumulation Cells = (Rate In - Rate Out) * Time,

where "rate in" equals the sum of the rate of initial cell attachment in the defined area and the rate that cells roll into the area and "rate out" is the sum of the rate of cell detachment

from the defined area, the rate that cells roll out of the area and the transmigration rate from the area. Taking the rate of transmigration to be negligible, equating equation (i) and (ii) and rearranging yields:

(iii) Rolling cells = [Rate attach + rate roll in - (rate detach + rate roll out)] * Time - Arrested cells

Thus, the above analysis reveals that the number of rolling cells on a specified surface area after a given time is a function of multiple factors, not solely the rate of attachment.

Another technique, referred to as a detachment assay, has also been used to study leukocyte adhesion under defined shear stress (4, 6, 27, 28). Cells are allowed to settle on a surface under static conditions for a few minutes. Buffer is then perfused over the surface beginning at a minimal flow rate which is subsequently increased in small increments after a given time period (usually about thirty seconds) at each flow rate. Immediately previous to each increase in flow rate, the number of cells adherent to the surface are tabulated. This method has been used widely to measure a cell's resistance to detachment from a surface under shear stress, and Lawrence et. al (28) used it to differentiate the role of L-selectin in neutrophil rolling from that in neutrophil attachment.

PSGL-1 has been intensely studied recently. The role of PSGL-1 in mediating neutrophil adhesion to P- and E-selectin has been examined by comparing adhesion to transfected Chinese hamster ovary (CHO) (20, 27) cells or immobilized substrates (27) in the presence or absence of PL-1, a murine monoclonal antibody (mAb) that blocks an epitope on PSGL-1 (20). Under static conditions, PSGL-1 is necessary for neutrophil adhesion to CHO cells transfected with P-selectin (CHO-P) (20, 27) and immobilized recombinant P-selectin (20), but not to CHO cells stably expressing E-selectin (CHO-E) (27).

Flow studies examining the role of PSGL-1 in mediating neutrophil adhesion under shear stress have measured the number of rolling cells on substrates consisting of CHO-P (20, 27) or CHO-E (27) cells or immobilized soluble P-selectin (20, 27). Assuming that the number of firmly arrested neutrophils is negligible, a reasonable assumption on a substrate solely expressing P- (29) or E-selectin, equation (i) reveals that the number of rolling cells should reflect the accumulation of cells. This method does not, however, allow for the differentiation of the ligand's capability to mediate rolling from that of attaching because if a cell is blocked from attaching, it lacks the opportunity to roll. It has been shown that flowing neutrophils require PSGL-1 in order to accumulate on CHO-P (20, 27). The role of PSGL-1 in neutrophil adhesion to E-selectin under flow, however, is less clear. Patel et. al (27) reported that PL-1 inhibits approximately 50% of neutrophil accumulation on CHO-E monolayers and concluded that PSGL-1 is required for optimal attachment to CHO-E. In contrast, Alon et. al (14) have recently shown that PL-1 does not affect accumulation on soluble E-selectin, suggesting that the putative binding site on PSGL-1 is not a major counter receptor for E-selectin.

Perhaps, the presence of L-selectin (25), in proximity to PSGL-1 (20) on neutrophil microvilli, confounds the identification of PSGL-1's contribution to the initial adhesive interactions of a neutrophil with vascular endothelium. Flow studies investigating the role of L-selectin in leukocyte - leukocyte interactions have provided insight into the function of the ligand. Bargatze et. al (15) have shown that neutrophils adherent to IL-1 activated HUVEC (human umbilical vein endothelial cell) monolayers support L-selectin-dependent neutrophil rolling. The authors suggested that these inter-leukocyte interactions may serve to augment leukocyte recruitment to sites of inflammation (15).

Recently, it has been shown that DREG-200, an anti-L-selectin mAb, or PL1 blocks 93% or 60%, respectively, of neutrophil rolling on neutrophils (30). Furthermore, PL1 or DREG-200 abolishes neutrophil rolling on purified neutrophil PSGL-1 (30). These results suggest that L-selectin and PSGL-1 can function as a receptor - ligand pair to mediate neutrophil attachment to neutrophils (30).

In another recent study, Alon et. al (14) conceptually divided the initial attachment or tether step (Step I) of the adhesion cascade into primary and secondary tethers. A primary tether event occurs when a tethering leukocyte's initial contact point is with the monolayer (14). A secondary tether occurs when a leukocyte initially interacts with an adherent leukocyte and subsequently attaches to the monolayer, usually downstream from adherent leukocytes in a linear formation aligned parallel to the flow direction (14, 15, 30). These secondary tethers are responsible for 70% of neutrophil accumulation on soluble P- or E-selectin substrates (14). Pretreating neutrophils with a L-selectin mAb DREG-56 does not significantly affect primary attachment to soluble E- or P-selectin but dramatically reduces secondary attachment to these substrates (14). These findings suggest that L-selectin is critical for attachment of flowing neutrophils to neutrophils already adherent to substrates expressing E- or P-selectin (14).

Numerous previous studies have examined the contribution of L-selectin to neutrophil adhesion to E- or P-selectin under flow without differentiating primary from secondary attachment events. DREG-56 treatment has been shown to significantly inhibit neutrophil accumulation on soluble E-selectin while having no affect on the number of rolling neutrophils on soluble E-selectin in a detachment assay (28). In addition, DREG-56 inhibits neutrophil accumulation on CHO-E monolayers by 70% (27) and on histamine stimulated HUVEC monolayers, which express P-selectin at high levels, by approximately 50% (29). These results are congruent with the recent findings of Alon et. al (14)

described above. However, in contrast with this recent study (14), Patel and co-workers (27) have reported that DREG-56 pretreatment does not affect neutrophil accumulation to CHO-P monolayers. Taken together, the results suggest that L-selectin is necessary for secondary leukocyte tethering but not for primary tethering nor rolling on E-selectin while the contribution of L-selectin to leukocyte accumulation on P-selectin remains to be clearly established.

Additionally, the function of L-selectin has been studied using neutrophils that have shed this ligand. fMLP activation of neutrophils, pretreated with cytochalasin D to prevent cell shape change or redistribution of P-selectin ligands (31), results in L-selectin shedding without loss of functional P-selectin static binding (27, 31). The accumulation of neutrophils treated in this manner is abolished on CHO-E and significantly reduced (~90%) on CHO-P monolayers (27). This data suggests that PSGL-1 is not sufficient for mediating neutrophil attachment to CHO-E or CHO-P and contrasts with the lack of effect on neutrophil accumulation on CHO-P monolayers observed, by the same investigators, with DREG pretreatment, as described above (27). The authors theorized that perhaps this incongruity stems from other activation induced changes to the neutrophil beyond simple L-selectin shedding. In sum, L-selectin plays a major role in accumulation of flowing neutrophils on substrates expressing E-selectin while studies of its contribution to accumulation on P-selectin have significantly different results.

Another potential source of confusion is inherent in the methods employed to isolate the contribution of an individual ligand in mediating leukocyte adhesion to P- or E-selectin. As described above, these methods have been strictly "subtractive" i.e. the function of a ligand is assigned by studying the behavior of a cell while blocking the ligand with a mAb, inducing it to be shed or cleaving it with an enzyme. Studies with mAbs may be complicated because an antibody may sterically hinder other binding ligands or even

activate the cell (32). In addition, activating cells to induce shedding may have multiple other effects on the cells, and enzymes may cleave more than the targeted ligand. Furthermore, "subtractive" methods are limited because although they can be used to determine if a ligand is necessary for a particular function, they are not able to address whether a ligand is sufficient. Thus, it has not been conclusively determined whether PSGL-1 is sufficient for mediating attachment to or rolling on P- or E-selectin under flow, and, in fact, some of the above results suggest that PSGL-1 is not sufficient to mediate attachment to CHO-P or CHO-E monolayers under flow (27).

Previous studies have focused on the function of specific domains of PSGL-1 in mediating adhesion to P- and E-selectin under static conditions. The amino terminal 19 amino acids of mature PACE (paired basic amino acid converting enzymes) - cleaved PSGL-1 consist of: (1) three tyrosines in proximity (21), at least one of which is sulfated (21, 33); (2) acidic residues flanking the tyrosines (21); and (3) threonines at positions 3 and 16 which are potential sites for O-linked oligosaccharides (21, 34). No N-linked glycosylation sites are located within this region (21). Microtiter wells coated with a chimera consisting of the N-terminal 19 amino acids of PSGL-1 fused to the heavy chain CH2-CH3 region of human IgG_1 Fc (19.Fc construct) have been shown to support adhesion of CHO-P or CHO-E cells (34). Binding of CHO-P cells, but not CHO-E cells, requires that PSGL-1 retains its N-terminal tyrosines and sulfation of at least one of the tyrosines (34). The function of the domains of PSGL-1 in mediating adhesion under flow remains to be determined.

The role of sialylated moieties in neutrophil adhesion to P- and E-selectin has been the subject of much investigation. The enzyme neuraminidase from *Vibrio cholerae* (VC) cleaves α2-3-, α2-6- and α2-8-linked sialic acids (35). VC neuraminidase pretreatment significantly inhibits neutrophil adhesion to radiolabeled P-selectin under static conditions

(17). Similarly, adhesion of neuraminidase-treated neutrophils to L cells expressing E-selectin is greatly diminished (36). The results under flow conditions also indicate that sialylated moieties are necessary for myeloid cell adhesion to P- or E-selectin. Pretreatment of flowing neutrophils with neuraminidase significantly inhibits their accumulation on soluble E-selectin (28) and on L-cells transfected with E-selectin (36). The human myeloid cell line HL60, which expresses PSGL-1 but not L-selectin, has been shown to bind P- and E-selectin under static (37) and flow (27) conditions. However, HL60 cells treated with VC neuraminidase at 37°C do not attach to P-selectin under flow (22).

Although PSGL-1 carries only a very small fraction of the total sLe^x on neutrophil membranes (19), it appears that the sLe^x on PSGL-1 is crucial for the ligand's activity under static conditions. Sialidase treatment of radiolabeled PSGL-1 abolishes binding to immobilized soluble P-selectin (24). Sako et. al (34) have shown that a mutant form of the 19.Fc construct with an alanine residue replacing the threonine at position 16, Thr-16, supports neither CHO-P nor CHO-E cell adhesion. In comparison to the 19.Fc construct, this mutant exhibits a shift in gel mobility which is consistent with loss of an oligosaccharide. The authors suggested that the threonine bears a sLe^x-modified O-linked oligosaccharide which is necessary for 19.Fc chimera binding to P- or E-selectin under static conditions. Moreover, recent mass spectroscopy and NMR data indicate that, of the N-terminal 19 amino acids of PSGL-1, only Thr-16 is glycosylated and that this glycan contains sLe^x (personal communication, Ray T. Camphausen, Genetics Institute, Cambridge, MA). Although sLe^x appears to be essential for PSGL-1 activity under static conditions, the function of sialylated structures on PSGL-1 under flow conditions is not known.

We wanted to determine whether PSGL-1 is sufficient to mediate attachment to and rolling on E- and P-selectin and if so, to characterize the domains of PSGL-1 that are

necessary or sufficient for these interactions. To address these issues, we studied the adhesion of 10 μm diameter nondeformable microspheres, coated with various extracellular regions of PSGL-1, to TNF-α activated HUVEC and CHO-E and CHO-P monolayers under defined flow conditions in a parallel plate chamber. The results demonstrate that: (1) the N-terminal 148 amino acids of PSGL-1 are sufficient to mediate microsphere attachment and rolling on TNF-α activated endothelial monolayers, CHO-E and CHO-P monolayers; (2) the 19 N-terminal amino acids of mature PSGL-1 are sufficient for attachment to and rolling on both E- and P-selectin; (3) within this 19 amino acid region, a sLe^x-containing glycan on threonine 16 is critical for attachment to E- and P-selectin and sustained low velocity rolling on P-selectin; (4) the N-terminal amino acid sequence 5 through 11, encompassing the three N-terminal tyrosines and flanking acidic residues, is necessary for attachment of the N-terminal 148 amino acids to P-selectin, but not to E-selectin under flow; and (5) PSGL-1 has one or more sites sufficient to support attachment to E-selectin, one at Thr-16 and one (or more) site(s) located between residues 20 through 148. Taken together, the results indicate that the properties of PSGL-1, which allow for formation of bonds with E- and P-selectin capable of supporting attachment and rolling, are not solely dictated by the mechanical properties of the leukocytes or microvilli which present PSGL-1. Rather, these bonds are at least partially a function of the molecular structure of PSGL-1.

Materials and Methods

Materials: RPMI-1640 containing 1 mM L-glutamine and 20 mM HEPES, Alpha media, DPBS with (DPBS+) or without Ca^{2+} and Mg^{2+}, were obtained from Biowhittaker (Walkersville, MD). FBS and dialyzed FBS were obtained from Hyclone (Urem, UT). O-sialoglycoprotein endopeptidase (OSGE) was obtained from Accurate Chemicals (Westbury, NY). Neuraminidase from *Vibrio cholerae* was obtained from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). All other chemicals were of the highest grade available from J.T. Baker Inc. (Phillipsburg, NJ).

Antibodies: Function blocking murine mAb to E-selectin 7A9 (IgG_1) was obtained from ATCC (clone HB 10135) and used as $F(ab')_2$ (10 μg/ml). Non-blocking murine mAb to E-selectin H4/18 (IgG_1) was used as $F(ab')_2$ (10 μg/ml) (12). Leukocyte function blocking murine anti-P-selectin mAb, HPDG2/3 (IgG_1) (21), was used as $F(ab')_2$ (10 μg/ml), non-blocking murine anti-P-selectin mAb, HPDG2/1 (IgG_1) (21) was used as purified IgG_1 (10 μg/ml). The following antibodies recognize human PSGL-1 and have been described previously: murine mAb PSL-275 (IgG_1; purified IgG, 20 μg/ml) (21, 33), and polyclonal rabbit sera, Rb3026 (1:20 dilution) (21, 33). Normal rabbit sera (1:20 dilution) was used as a control for Rb3026. mAbs KPL1 and KPL2 (K.R. Snapp et al., submitted for publication) recognize PSGL-1 and were used as ascites (1:200 dilution for adhesion assays and 1:500 for flow cytometric analysis) and purified IgG. Murine anti-human IgG_1 Fc specific (Caltag Laboratories; San Francisco, CA) was used a control for the purified KPL1. Murine anti-ICAM-1 mAb Hu5/3 (IgG_1) was used as purified IgG (20 μg/ml) (2), anti-sLe^x mAb CSLEX (Becton Dickinson; San Jose, CA) (IgM) was used as purified IgM (20 μg/ml). Negative control for CSLEX was purified murine IgM (Sigma).

Murine antibody to human Class I, W6/32, (IgG_{2a}) was used as $F(ab')_2$ (10 μg/ml) (2, 12). FITC labeled goat $F(ab')_2$ secondary antibodies (each at 1:50 dilution) to rabbit IgG, mouse IgG, mouse IgM and human Fc were obtained from Caltag Laboratories. FITC labeled goat $F(ab')_2$ secondary antibody (1:50 dilution) to mouse Fc and unlabeled goat $F(ab')_2$ were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Cell culture. Chinese hamster ovary (CHO) cells stably expressing human E-selectin (CHO-E) or human P-selectin (CHO-P) were prepared and cultured in Alpha media containing 10% dialyzed FBS as previously detailed (21). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously (2). For induction of adhesion molecule expression, HUVEC monolayers were activated by 4 hour treatment with 25 ng/ml TNF-α (38).

Soluble forms of PSGL-1: The PSGL-1 molecules used in these studies are chimeric constructs consisting of various truncated extracellular domains of mature recombinant PSGL-1 fused to the heavy chain CH2-CH3 (Fc) region of human IgG_1 (Figure 4A). They are available through a collaborative agreement between Dr. F. W. Luscinskas of Brigham and Women's Hospital and Dr. Ray T. Camphausen of the Genetics Institute in Cambridge, MA. The construction, expression and selectin binding properties of the chimeras have been described earlier (34). Two of the chimeras used in the present studies (Figure 4B) were previously synthesized by transfecting plasmids encoding the 148.Fc or the ΔY.148.Fc chimeras, into COS cells as described, except for the substitution of a plasmid encoding an α(1,3)fucosyltransferase-VII (Fuc-TVII) enzyme (39, 40) for the Fuc-TIII plasmid used in earlier cotransfections (34). Another chimera used in the present study consists of the 19.Fc form of PSGL-1 described earlier (34) that was mutated to include an enterokinase cleavage site (41) located between the PSGL-1 and IgG_1 Fc domains and is termed 19.ek.Fc (Figure 4B). A plasmid encoding this construct was

stably transfected into CHO cells which were engineered to also express Fuc-TVII and β(1,6)-N-acetylglucosaminyltransferase ("core2", (42)) activities (43, 44). Similar to COS produced 19.Fc, 19.ek.Fc is tyrosine sulfated and glycosylated with sLe^x-type containing O-glycans and, in E- and P-selectin binding experiments (34), is functionally indistinguishable (Ray T. Camphausen, personal communication). The construction, expression and characterization of 19.ek.Fc will be described (Ray T. Camphausen, Kenneth M. Comess and Steven Howes, manuscript in preparation). The nomenclature for microspheres coated with various extracellular domains of PSGL-1 is as follows: the 148.Fc construct, 148.Fc microspheres; the 19.ek.Fc construct, 19.ek.Fc microspheres, the ΔY.148.Fc construct, ΔY.148.Fc microspheres (Figure 4B).

Preparation, flow cytometric analysis and enzymatic treatment of PSGL-1 microspheres.

Preparation: The protein A concentration necessary for saturated adsorption to 10 μm nondeformable polystyrene microspheres (Polysciences, Warrington, PA) was initially determined. Microspheres were washed twice in 0.1 M $NaHCO_3$, pH 9.2, and incubated (2.5×10^7 microspheres/ml) overnight at RT with increasing concentrations of FITC-labeled protein A (Zymed, South San Francisco, CA) at increasing concentrations in 0.1 M $NaHCO_3$, pH 9.2, with end to end rotation. Microspheres were then washed twice in DPBS and FITC fluorescence was determined as detailed below. Microspheres were found to saturate at approximately 30 μg/ml protein A (data not shown). To assure saturation of microspheres with protein A in the preparation of PSGL-1 microspheres, microspheres were prepared as in saturation experiments but incubated with 300 μg/ml of non-labeled, instead of labeled, protein A (Zymed, South San Francisco, CA). Microspheres were then washed twice with DPBS and blocked for 30 minutes at RT with DPBS containing 1% BSA. Following the blocking step, the microspheres (2×10^8 microspheres/ml) were incubated with a PSGL-1.Fc extracellular construct or human IgG_1

diluted in DPBS for 1 hour at RT with agitation (Figure 5). The microspheres were washed with DPBS+, 1% BSA and blocked with DPBS+, 1% BSA containing 200 μg/ml human IgG_1 kappa or lambda (Sigma). Microspheres were held in this buffer at 1 x 10^8 microspheres/ml at RT for 1 hr prior to use in the assays.

Indirect immunofluorescence and flow cytometric analysis of PSGL-1 microspheres:

Following the human IgG_1 blocking step, replicate aliquots (~5 x 10^5) of microspheres were washed in DPBS+ containing 1% BSA and incubated for 20 minutes at RT with 20 μg/ml primary mAb in DPBS+, 1% BSA. After incubation, microspheres were washed and then blocked with unlabeled goat $F(ab')_2$ fragments (100 μg/ml), and the primary mAb was detected with FITC labeled $F(ab')_2$ fragments of goat anti-mouse Fc specific secondary antibody. The microspheres were incubated for 20 minutes at RT, washed twice in DPBS+, 1% BSA, once in DPBS+, and fixed with 2% formaldehyde in DPBS+. FITC fluorescence was determined on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by counting the fluorescence of 5,000 or 10,000 microspheres. The data are presented as single parameter histograms on a four decade scale.

O-Sialoglycoprotein endopeptidase treatment of PSGL-1 microspheres: Microspheres were prepared as described above prior to blocking with human IgG_1. A suspension of PSGL-1 coated microspheres (1 x 10^8/ml) was incubated in DPBS+, 0.2% FBS, 0.05% NaN_3, 25 mM HEPES, pH 7.4, with or without 160 μg/ml OSGE for 30 minutes at 37°C. The microspheres were then washed with DPBS+, 1% BSA and blocked with DPBS+, 1% BSA containing 200 μg/ml human IgG_1 at 1 x 10^8 microspheres/ml. The microspheres used in the flow cytometric analysis to detect human Fc were incubated with 0.2% normal rabbit serum instead of human IgG_1, blocked with 100 μg/ml unlabeled goat $(Fab')_2$

fragments and then stained with goat FITC labeled $(Fab')_2$ fragments of antibodies to either human Fc or mouse IgG.

Sialidase treatment of PSGL-1 microspheres: Microspheres were prepared with the 19.ek.Fc construct as described above. 19.ek.Fc microspheres were then washed twice in RPMI-1640, resuspended to 1 x 10^8 in RPMI-1640 with or without 0.1 U/ml neuraminidase and incubated for 30 minutes at 37 °C. Microspheres were next washed twice in DPBS+, 1% BSA and blocked (1 x 10^8 microspheres/ml) with DPBS+, 1% BSA containing 200 μg/ml human IgG_1. 148.Fc and 19.ek.Fc microspheres treated with CSLEX (murine IgM) and subsequently stained with FITC labeled $(Fab')_2$ fragments of goat anti-mouse IgM did not exhibit an increased fluorescence (by flow cytometric analyses) relative to microspheres treated with control, purified murine IgM (data not shown).

Parallel plate flow chamber analysis: The parallel plate flow chamber apparatus used in these studies (Figure 6) has been described in detail (2). Briefly, the chamber consists of two stainless steel plates separated by a silastic gasket. The coverslip covered with a cellular monolayer was placed in the flow chamber and fluid drawn through the chamber at defined flow rates using a syringe pump (model 44; Harvard Apparatus Inc., Natick, MA). Shear stresses at the adhesive surface were calculated from the equation $\tau = 3\mu Q/2ba^2$ where a is one-half the distance between the top and the bottom of the flow chamber, 0.125 mm, b is the width of the flow field, 5 mm, Q is the flow rate of the fluid drawn through the chamber, and μ is the viscosity of the fluid assumed to be the viscosity of water at 37°C, 0.7 cp. Temperature was maintained at 37°C by a heating plate. The flow apparatus was mounted on an inverted microscope (Nikon Diaphot) and the entire perfusion period was recorded on videotape by a video camera and VCR. Microspheres were drawn through the chamber at 5 x 10^5 microspheres/ml in DPBS+, 0.5% BSA and

microsphere attachment rates were quantified by observing a 10x field of view for approximately 1 minute and 15 seconds. The number of microspheres that attached throughout this time period was determined and divided by the time of observation and the area of the field of view to yield the rate of attachment per unit area. For a given coverslip, an attachment rate was determined at two different fields of view and the two values were averaged to give an n=1. Microsphere accumulation was determined by counting the number of microspheres interacting with the substrate in a particular field of view (10x magnification). This value was determined for 3-5 different fields of view between 3.5 to 4 minutes after initiating of microsphere perfusion. These numbers were averaged and normalized to the area of the field of view. Rolling velocities were determined manually by measuring the distance a microsphere traveled in a given amount of time.

Antibody blocking experiments: The CHO-E cell monolayers were treated with function blocking $F(ab')_2$ fragments of anti-E-selectin mAb 7A9, non function blocking anti-E-selectin mAb H4/18 or anti-Class I mAb W6/32. The CHO-P cell monolayers were treated with function blocking $F(ab')_2$ fragments of anti-P-selectin mAb HPDG2/3, non function blocking anti-P-selectin mAb HPDG2/1 or anti-Class I mAb W6/32. TNF-α stimulated HUVEC monolayers were treated with function blocking $F(ab')_2$ fragments of anti-E-selectin mAb 7A9, or anti-Class I mAb W6/32. All antibodies were diluted (10 μg/ml) in the appropriate media and added to the cell monolayers 20 minutes prior to the adhesion assays described above. For mAb blocking of the PSGL-1 microspheres, 10μl - 15 μl of a 1 x 10^8 microsphere/ml suspension was mixed 1:1 with DPBS+, 1% BSA containing a 1:100 dilution of ascites mAb KPL1 or KPL2. This suspension was allowed to incubate for 15 minutes at room temperature and then diluted to 5 x 10^5 microspheres/ml in DPBS+, 0.5% BSA and used immediately in the flow chamber assay.

Statistics: All differences were evaluated by a two-tailed Student's t-test. p values represent the results of these t-tests and values ≤ 0.05 were considered significant. All error bars represent standard deviations.

Results

PSGL-1 Fc chimera binds protein A-coated polystyrene microspheres in a dose-dependent and saturable fashion

Previous studies have used biotinylated sLe^x (45) or mAbs (46, 47) coupled to microspheres to model simplified cells in adhesion assays. We adapted this technique to determine whether PSGL-1, in isolation, is able to mediate adhesion to P- or E-selectin under flow conditions. Polystyrene microspheres with a diameter of 10 μm were used because their density (1.05 g/ml) and diameter approximate those of leukocytes.

Initially, the concentration of fluorescently labeled protein A necessary for saturated adsorption to microspheres was determined (data not shown). To assure saturating conditions in the preparation of PSGL-1 microspheres, non-labeled protein A was added to microspheres at a concentration approximately 10 fold greater than the predetermined saturating concentration. PSGL-1 molecules were then coupled to the microspheres via protein A (Figure 5). The PSGL-1 molecules used in these studies are chimeras consisting of truncated amino terminal extracellular segments of mature recombinant PSGL-1 fused via their most C-terminal end to the CH2-CH3 region of the Fc chain of human IgG_1 (Figure 4A, B). It has been shown that intact covalently linked CH2-CH3 Fc domain of IgG has a high affinity for protein A (48), and this property was utilized to couple chimeras to the protein A-coated microspheres. It is reasonable to assume that this synthesis process yields PSGL-1 microspheres with most of their PSGL-1 oriented such that the N-terminal region extends away from the microsphere (Figure 5). This orientation approximates that of neutrophil-bound PSGL-1, on the tips of microvilli and available for adhesion under flow (20).

The 148.Fc construct, composed of the N-terminal 148 amino acids of mature PSGL-1 fused to the Fc region of human IgG_1, was used to characterize the binding of PSGL-1 chimeras to protein A-coated microspheres. This construct was chosen because it binds with high affinity to P- and E-selectin under static conditions (34). To detect PSGL-1, the 148.Fc microspheres were treated with a mAb to PSGL-1, PSL275, followed by indirect immunofluorescence and flow cytometry. Binding of the 148.Fc construct to protein A-coated microspheres was dose-dependent and saturable (Figure 7, a-e). As a control for the Fc region of the construct, human IgG_1 was added to protein A-coated microspheres to form IgG_1 microspheres (Figure 5), and these microspheres had negligible FITC fluorescence when detected with PSL275 (data not shown). In addition, microspheres that were synthesized with 100μg/ml of the 148.Fc construct and detected with Hu5/3, a control non-binding isotype matched mAb that recognizes intercellular adhesion molecule-1 (ICAM-1) had negligible FITC fluorescence (Figure 7, f), confirming the specificity of the PSGL-1 - PSL275 interaction.

Preliminary flow studies with microspheres synthesized with 50 μg/ml of the 148.Fc construct yielded consistent adhesion results (data not shown). Hence, all subsequent experiments were conducted with this concentration of PSGL-1 constructs unless otherwise noted.

148.Fc microspheres attach to and roll on TNF-α activated HUVEC monolayers

In addition to inducing maximal E-selectin expression, 4 hour cytokine activation of HUVEC increases synthesis of vascular cell adhesion molecule-1 (VCAM-1) (49) and ICAM-1 (50, 51) and induces low level expression of P-selectin (38). Treatment of IL-1β activated HUVEC monolayers with a blocking mAb to E-selectin moderately reduces

accumulation of flowing neutrophils (~30%) (36). PSGL-1 has been implicated in neutrophil adhesion to CHO-E under flow (27), but to the best of our knowledge, PSGL-1 mediated adhesion to activated HUVEC has not been directly studied.

To address whether PSGL-1 is sufficient to mediate microsphere attachment to and rolling on stimulated HUVEC under flow conditions, we perfused 148.Fc microspheres over TNF-α activated HUVEC monolayers in a parallel plate flow chamber. At 2 dynes/cm^2, 148.Fc microspheres attached to 4 hour TNF-α activated HUVEC monolayers at a rate of 101 ± 14 microspheres/mm^2/min (Figure 8). Subsequent to attachment, greater than 95% of attached 148.Fc microspheres rolled in a manner similar to that described for leukocytes (Figure 9) on lipopolysaccharide (52) or histamine (29) stimulated HUVEC monolayers, i.e. a low velocity, high variance translation. Fewer than 5% of attached 148.Fc microspheres firmly arrested and none were observed to migrate through the endothelium. Treatment of HUVEC monolayers with F(ab')$_2$ fragments of function-blocking mAb to E-selectin, 7A9, eliminated adhesion while F(ab')$_2$ fragments of a control mAb to Class I, W6/32, had no affect on adhesion (Figure 8). IgG_1 microspheres, a control for the Fc region of the PSGL-1 chimera, failed to attach to activated HUVEC monolayers (Figure 8). Finally, 148.Fc microspheres did not attach to unactivated HUVEC monolayers (Figure 8). These results suggest that the N-terminal 148 amino acids of PSGL-1 are sufficient to mediate both microsphere attachment to TNF-α activated HUVEC monolayers via E-selectin and microsphere rolling on TNF-α activated HUVEC monolayers.

148.Fc microspheres attach to and roll on CHO-P and CHO-E monolayers

CHO-P and CHO-E monolayers were used to study the adhesion of PSGL-1 to P- and E-selectin, respectively, in the absence of other adhesion molecules found on activated

endothelium. Jones et. al (29) have recently shown that flowing neutrophils consistently accumulate on histamine stimulated primary passage HUVEC monolayers via neutrophil interaction with P-selectin. In the present studies passage one HUVEC were of limited supply, and preliminary experiments were conducted that showed highly variable expression of P-selectin on second passage HUVEC monolayers stimulated with histamine (data not shown). Thus, CHO-P monolayers were also used as a model of HUVEC monolayers expressing P-selectin.

To determine if PSGL-1 is sufficient to mediate attachment and rolling on P- and E-selectin, we studied the interaction of 148.Fc microspheres with CHO-P and CHO-E monolayers in the parallel plate flow chamber. At 2 dynes/cm^2, 148.Fc microspheres attached to CHO-E and CHO-P monolayers at a rate of 122 ± 50 microspheres/mm^2/min and 119 ± 43 microspheres/mm^2/min, respectively (Figure 10). Similar to the results on activated HUVEC, subsequent to attachment to either CHO-E or CHO-P monolayers, > 95% of the attached 148.Fc microspheres rolled, <5% firmly arrested and none were observed to transmigrate. Function blocking F(ab')$_2$ mAbs to E- (7A9) and P-selectin (HPDG2/3) completely inhibited the attachment of 148.Fc microspheres to CHO-E and CHO-P monolayers, respectively (Figures 10). In contrast, control non-binding F(ab')$_2$ preparation of mAb W6/32 (Figure 10) and non-function blocking isotype matched mAbs to E- (H4/18) and P-selectin (HPDG2/1) (data not shown) had no affect on the rate of attachment of 148.Fc microspheres to CHO-E and CHO-P monolayers, respectively. Under identical conditions, the 148.Fc microspheres did not attach to the parental CHO cell line (n = 2, data not shown), nor did human IgG$_1$ microspheres attach to CHO-P or CHO-E monolayers (Figures 10). Thus, it appears that the first 148 N-terminal amino acids of PSGL-1 are sufficient to support attachment to and subsequent rolling on CHO cell monolayers expressing E- or P-selectin.

O-sialoglycoprotein endopeptidase pretreatment abolishes 148.Fc microsphere attachment to CHO-E and CHO-P monolayers

O-sialoglycoprotein endopeptidase (OSGE) from *Pasteurella haemolytica* is a metalloprotease that selectively cleaves clustered O-linked mucin-like glycoproteins (53), such as PSGL-1 (19, 20, 22) and abolishes static PSGL-1 (20) and HL60 cell (19, 54) adhesion to P-selectin. Under flow conditions, OSGE eliminates HL60 attachment to soluble P-selectin (22) and rolling HL60 cells on CHO-P (27). However, the data on E-selectin is less consistent as OSGE treatment reduces the number of rolling HL60 cells on CHO-E by 50% (27) but does not affect the attachment rate on soluble E-selectin (22).

To further investigate the affect of OSGE treatment on PSGL-1 mediated interactions with E-selectin and to evaluate the specificity of 148.Fc microsphere attachment events, 148.Fc microspheres were treated with OSGE and then used in adhesion flow assays. OSGE treatment significantly reduced (>95%) the presence of an epitope required for PSGL-1 mAb PSL275 binding (Figure 11A). Additionally, FITC labeled $F(ab')_2$ fragments of antibodies to human Fc confirmed that OSGE cleaved the PSGL-1 portion, not the Fc region, of 148.Fc microspheres (Figure 11A). In the parallel plate flow chamber, 148.Fc microspheres pretreated with OSGE failed to attach to CHO-E and CHO-P monolayers (Figure 11B). These results indicate that the 148.Fc construct requires the PSGL-1 region to attach to CHO-E and CHO-P.

The function of PSGL-1 regions in mediating microsphere attachment to and rolling on CHO-E and CHO-P monolayers

Under static conditions it has been shown that the 19 N-terminal amino acids of PSGL-1 are sufficient to mediate PSGL-1 adherence to P- or E-selectin (34) and that at

least one N-terminal sulfated tyrosine is necessary for adherence to P- but not to E-selectin (33, 34). To characterize the function of these PSGL-1 regions, Sako et. al (34) used a variety of PSGL-1.Fc constructs, composed of mutated or intact amino-terminal segments of PSGL-1 fused to the CH2-CH3 domain of human IgG_1 Fc (Figure 4A). In addition to the 148.Fc construct, this previous study relied heavily on the ΔY.148.Fc and 19.Fc constructs (Figure 4B) (34). The ΔY.148.Fc construct is a mutated form of the 148.Fc construct with an internal deletion of N-terminal amino acids in positions 5-11 which includes three tyrosines and flanking acidic residues (Figure 4B). The PSGL-1 portion of the 19.Fc construct is comprised of the N-terminal 19 amino acids of mature PSGL-1 (Figure 4B).

To delineate the function of PSGL-1 regions under flow, we used 148.Fc, ΔY.148.Fc and 19.ek.Fc microspheres. The 19.ek.Fc microspheres were constructed from the 19.ek.Fc construct which is a modified form of the 19.Fc construct with an enterokinase cleavage site inserted between the PSGL-1 and Fc domains (Figure 4B). The ΔY.148.Fc construct has a similar molecular weight as the 148.Fc construct (34) and, because of material limitations, was coupled to microspheres at a concentration of 40 μg/ml. For these experiments, 148.Fc microspheres were also prepared with 40 μg/ml. Since the molecular weight of the 19.ek.Fc construct is a little over 1/3 that of the 148.Fc construct, we prepared the 19.ek.Fc microspheres with 15 μg/ml of the 19.ek.Fc construct. As shown with a polyclonal antibody to PSGL-1, Rb3026, all constructs were coupled to the microspheres (Table 1).

In the parallel plate flow chamber at 2 dynes/cm^2, 19.ek.Fc microspheres attached to CHO-E and CHO-P monolayers at a rate similar to that observed for 148.Fc microspheres (Figure 12A). The ΔY.148.Fc microspheres attached to CHO-E monolayers at a rate similar to that observed for 148.Fc microspheres, but did not attach to CHO-P

monolayers (Figure 12A). Subsequent to attachment, > 95% of the 19.ek.Fc microspheres rolled on CHO-E or CHO-P monolayers and >95% of the ΔY.148.Fc microspheres rolled on CHO-E monolayers. Neither the 19.ek.Fc nor the ΔY.148.Fc microspheres attached to the parental CHO cell line (n=2; data not shown).

To provide further insight into the function of specific PSGL-1 domains, prior to flow assays, PSGL-1 microspheres were treated with KPL1, a mAb to PSGL-1 which blocks neutrophil and memory T-cell adhesion to CHO-P monolayers, or with KPL2, a non-blocking isotype matched mAb to PSGL-1 (K.R. Snapp and G.S. Kansas, personal communication). KPL1 recognition of the 148.Fc microspheres appears to require the N-terminal amino acid sequence 5 through 11 of PSGL-1 while KPL2 does not recognize any of the microspheres (Table 1). Thus, KPL2 was a non-binding control mAb in the microsphere experiments. KPL1 did not block 148.Fc microsphere attachment to CHO-E monolayers, but eliminated attachment to CHO-P monolayers (Figure 12B). In contrast, KPL1 abolished 19.ek.Fc microsphere attachment to both CHO-E and CHO-P monolayers (Figure 12C). Ascites preparations of the antibodies were used in these blocking experiments and similar results were obtained with a purified IgG preparation of KPL1 (data not shown). Moreover, relative to pretreatment with KPL2, attachment of 148.Fc microspheres to CHO-E monolayers was not inhibited by prior treatment with a higher concentration of an ascites preparation of KPL1 (1:100 dilution vs. 1:200 dilution) or 80 μg/ml of purified KPL1 (data not shown).

These results indicate that: (1) the 19 N-terminal amino acids of PSGL-1 are sufficient to support attachment and rolling on both CHO-P and CHO-E monolayers; (2) the amino acid sequence 5 through 11 of PSGL-1, consisting of acidic residues and at least one sulfated tyrosine, is necessary for the N-terminal 148 amino acids to support attachment to CHO-P monolayers, but not CHO-E, monolayers; and (3) the N-terminal 148

amino acids contain at least two sites that are sufficient to support attachment to CHO-E monolayers, one (or more) within the first N-terminal 19 amino acids and a second (or more) site(s) located on the carboxyl terminal side of amino acid 19.

Sialylated structures on 19.ek.Fc microspheres are necessary for attachment to CHO-E monolayers and for sustained low velocity rolling and accumulation on CHO-P monolayers

The N-terminal 19 amino acids of PSGL-1 require the presence of a sLe^x-modified O-linked oligosaccharide on the threonine at position 16, Thr-16, for static adhesion to P- or E-selectin (34 and Ray T. Camphausen, personal communication). Under flow conditions, pretreatment of myeloid cells with 0.1 U/ml neuraminidase for 30 minutes at 37°C prevents their accumulation on P- (22) or E-selectin (28, 36). Because neutrophils display multiple glycosylated proteins and lipids and PSGL-1 carries only a small percentage of total neutrophil membrane sLe^x (19), it is difficult to predict the function of sialylated structures on PSGL-1 under flow from these previous studies. Thus, to investigate the role of sialylated structures in PSGL-1 mediated attachment and rolling on CHO-P and CHO-E monolayers, 19.ek.Fc microspheres were treated with neuraminidase prior to flow assays.

Preliminary flow studies showed that neuraminidase pretreatment (0.1 U/ml, 37°C for 30 minutes) of microspheres coated with 15 μg/ml of the 19.ek.Fc construct reduced the rate of attachment to CHO-E monolayers by > 95% and to CHO-P monolayers by > 85% (data not shown). 19.ek.Fc microspheres were then prepared with 50 μg/ml 19.ek.Fc rather than 15 μg/ml and treated with neuraminidase according to the same protocol. Treatment of 50 μg/ml 19.ek.Fc microspheres with neuraminidase essentially eliminated attachment to CHO-E monolayers (Figure 13a). In contrast, these 19.ek.Fc microspheres treated with neuraminidase attached to CHO-P monolayers at a rate similar to

untreated 19.ek.Fc microspheres (Figure 13b). Hence, a higher level of sialylation is required for the 19.ek.Fc microspheres to attach to CHO-E relative to CHO-P monolayers.

Neuraminidase pretreatment markedly changed 50 μg/ml 19.ek.Fc microsphere behavior subsequent to attaching to CHO-P monolayers. Typically, these 19.ek.Fc microspheres attached to CHO-P monolayers, then rolled and skipped for several microsphere diameters before finally detaching. The increased rate of detachment resulted in a decrease in the accumulation relative to untreated 19.ek.Fc microspheres (Figure 13c). The rolling velocities on CHO-P monolayers increased five-fold, from 2 μm/sec for untreated 19.ek.Fc microspheres to 10 μm/sec with neuraminidase treatment ($p < 0.05$; $n = 10$ for each condition).

Conclusion

Leukocyte extravasation from the vascular compartment is a highly regulated process that is controlled largely by the complex leukocyte - endothelial adhesion cascade involving multiple receptor - ligand interactions. PSGL-1 is a mucin-like glycoprotein found on the surface of leukocytes that has been implicated in the accumulation of flowing neutrophils to P-(20, 27) or E-selectin (27). The seminal studies of PSGL-1 on neutrophils have assigned function by comparing neutrophil adhesion to transfected CHO cells or immobilized substrates in the presence or absence of PL1, a function blocking mAb to PSGL-1 (20, 27). But, it is possible that PL-1 sterically hinders other adhesion molecules on the neutrophil surface, such as L-selectin, and consequently, this method may overestimate PSGL-1's binding capability. Moreover, this method does not allow for the investigation of whether PSGL-1 is sufficient for a function. It also does not permit study of PSGL-1's contribution to rolling because if a cell is blocked from attaching, it lacks the opportunity to roll. The focus of our work has been to determine whether PSGL-1 is sufficient to mediate attachment and rolling on P- and E-selectin and to characterize the regions of PSGL-1 that are necessary and/or sufficient for these interactions.

To address these issues, we initially coupled the N-terminal 148 amino acids of mature PSGL-1 to inert polystyrene microspheres and evaluated the interaction of these 148.Fc microspheres under flow conditions with 4 hour TNF-α activated HUVEC monolayers which express high levels of E-selectin and with monolayers composed of CHO cells transfected with E- or P-selectin. Our findings indicate that the N-terminal 148 amino acids of PSGL-1 are sufficient to mediate attachment to and rolling on each of these monolayers. The attachment step requires E-selectin expression on the activated HUVEC and the CHO-E monolayers and P-selectin on the CHO-P monolayers. To the best of our

knowledge, the present study is the first to examine the specific contribution of PSGL-1 in adhesion to the physiologically relevant cytokine activated HUVEC monolayers.

To investigate the minimal PSGL-1 motif(s) necessary and/or sufficient for the ligand to form physiologically relevant bonds with E- or P-selectin, we evaluated the interaction of flowing 19.ek.Fc microspheres with CHO-P and CHO-E monolayers at 2 dynes/cm^2. The 19.ek.Fc construct used to synthesize these microspheres consists of the 19 N-terminal amino acids of PSGL-1 linked to human IgG_1 Fc via an intermediary enterokinase cleavage site (Figure 4B). The 19 N-terminal amino acids were chosen because they have been shown to adhere to microtiter wells coated with CHO-E or CHO-P cells (34). This PSGL-1 sequence includes an anionic polypeptide segment and two potential O-linked glycosylation sites with threonines at positions 3 and 16, but no potential N-linked glycosylation sites (21). The anionic polypeptide segment consists of three tyrosines (21), at least one of which is sulfated (33, 34), and flanking acidic residues (21). Within the N-terminal 19 amino acids, Thr-16 is the only residue modified and this glycan contains sLe^x as assessed by Ray T. Camphausen at Genetics Institute, Cambridge, MA. Our findings indicate that the 19 N-terminal amino acid sequence, comprised of two established static adhesion components (34), an anionic polypeptide with at least one sulfated tyrosine and an O-linked sLe^x modified oligosaccharide, is sufficient to mediate attachment to and rolling on CHO-E or CHO-P cells under flow conditions.

We then further dissected the function of these minimal PSGL-1 adhesion regions under flow. Under static conditions, the anionic polypeptide segment is necessary for PSGL-1 adhesion to P-selectin but not to E-selectin (34). As might be predicted, our results indicate that PSGL-1 requires this segment to attach to CHO-P monolayers under flow (Figure 12A). However, our findings on CHO-E monolayers are more complex. Flow studies with the ΔY.148.Fc microsphere, a mutated form of the 148.Fc microsphere

with an internal deletion of the N-terminal anionic polypeptide, indicate that the N-terminal 148 amino acids do not require this polypeptide to attach to CHO-E monolayers under flow (Figure 12A). We found that KPL1, a mAb to PSGL-1 that blocks neutrophil and memory T-cell adhesion to CHO-P monolayers (K.R. Snapp and G.S. Kansas, personal communication), requires the anionic polypeptide to bind PSGL-1 (Table 1). In agreement with our ΔY.148.Fc microsphere data, this antibody has no affect on 148.Fc microsphere attachment to or rolling on CHO-E monolayers (Figure 12B). However, KPL1 eliminates 19.ek.Fc microsphere attachment to CHO-E (Figure 12C). Thus, PSGL-1 contains at least two binding sites for E-selectin, one (or more) located within the first 19 amino acids and a second (or more) site(s) C-terminal to amino acid 19.

The markedly different affect of KPL1 pretreatment on the interaction of flowing 148.Fc and 19.ek.Fc microspheres with CHO-E monolayers is striking. A possible explanation for this difference is that the 19.ek.Fc microspheres indeed require the anionic polypeptide segment to attach to CHO-E monolayers. In this study, we found that sialylated moieties are necessary for N-terminal 19 amino acids of PSGL-1 to bind to E-selectin under flow, and it is conceivable that a certain amount of sialylated structures and anionic polypeptides are necessary to reach a threshold (perhaps of negative charge) for E-selectin binding. In contrast to the 148.Fc microspheres which most likely bear numerous sialylated structures (18, 21, 24, 34), 19.ek.Fc microspheres carry a single sLe^x-modified O-linked oligosaccharide. Thus, the 19.ek.Fc microspheres may require the N-terminal anionic polypeptide to reach the putative E-selectin binding threshold while this polypeptide may be superfluous for the 148.Fc microspheres. This schema, however, would not be predicted from previous static assays which have shown that a mutated form of the 19.Fc construct, with phenylalanines replacing the three N-terminal tyrosines, has a significant residual level of E-selectin binding (34). This finding provides evidence that the anionic

polypeptide is, in fact, not crucial for E-selectin binding under static conditions and instead implies the importance of the sialylated moiety.

Consequently, the following explanation for the different behavior of KPL1 treated 19.ek.Fc and 148.Fc microspheres on CHO-E monolayers appears more likely. KPL1 requires the amino acids in positions 5-11 to bind PSGL.Fc microspheres. This amino acid sequence is in proximity to Thr-16 which bears the only sialylated structure within the 19 N-terminal amino acids. It is possible that KPL1 sterically hinders this sialylated structure and thus, depletes the 19.ek.Fc microspheres of functional sialylated moieties. In contrast, 148.Fc microspheres have numerous other potential sites for sLe^x-modified O-linked oligosaccharides. Because sialylated moieties are required for PSGL-1 binding to E-selectin (Figure 13a), steric hindrance is a plausible explanation for KPL1 treatment abolishing 19.ek.Fc attachment while having no affect on 148.Fc microsphere attachment to CHO-E monolayers.

Recently, it has been shown that microspheres derivitized with sLe^x attach and roll on E-selectin, suggesting that sLe^x is sufficient for E-selectin binding under flow (45). In preliminary experiments, we found that neuraminidase pretreatment of microspheres coated with 15 μg/ml of 19.ek.Fc construct significantly reduced attachment to CHO-E (>95%) and CHO-P monolayers (>85%). These data suggest that sialylated glycans are necessary for attachment to CHO-E and CHO-P monolayers. Further experiments revealed that identical neuraminidase pretreatment of microspheres coated with 50 μg/ml of the 19.ek.Fc construct eliminated attachment to CHO-E monolayers (Figure 13a), but had no affect on attachment to CHO-P monolayers (Figure 13b). It is likely that desialylation of the oligosaccharide linked to Thr-16 on these 50 μg/ml 19.ek.Fc microspheres was incomplete. The residual sialylated glycan was not capable of mediating attachment to E-selectin but, in conjunction with the N-terminal anionic polypeptide, supported attachment

to P-selectin. Thus, a higher level of sialylation is required for attachment to E-selectin than to P-selectin. In contrast to the > 95% of attached untreated 19.ek microspheres which rolled at sustained low velocity on the transfected CHO monolayers, neuraminidase treated 19.ek microspheres typically attached to CHO-P monolayers, then skipped and rolled at high velocity for a distance on the order of 100 μm before detaching. We conclude that an elevated sialylation level is required for sustained low velocity translation on P-selectin whereas attachment to P-selectin occurs at a lower density.

Previous flow studies have shown that OSGE treatment abolishes HL60 interaction with P-selectin (22, 27), while the results on E-selectin are more complex. OSGE treatment decreases the number of rolling HL60 cells on CHO-E monolayers by 50% (27) while not affecting HL60 attachment to soluble E-selectin substrates (22). It is helpful to refer to equation (iii) in interpreting this adhesion data on substrates expressing E-selectin. The following interpretation assumes that the difference between the soluble E-selectin and CHO-E substrates is insignificant in comparison to the large difference between the effect of OSGE treatment on the number of rolling HL60 cells (~50% decrease) and the lack of effect on attachment rate (~0% change). Assuming that the rate of transmigration and number of arrested cells are negligible and the average rolling velocity is constant, so that rate of cells rolling into the defined area approximates the rate of cells rolling out, equation (iii) reduces to:

(iv) Rolling cells = [Rate attach - rate detach] * Time

Thus, the unchanged HL60 cell attachment rate but decrease in number of rolling cells on E-selectin with OSGE treatment may result from an increase in the rate of detachment. These findings suggest that OSGE-sensitive ligands may mediate HL60 cell sustained rolling and prevent detachment on E-selectin substrates.

In conjunction with these previous studies (22, 27), our results with OSGE treated microspheres suggest that myeloid cells have at least one OSGE-resistant binding site independent of the N-terminal 148 amino acids of PSGL-1 that is sufficient to mediate attachment to CHO-E monolayers under flow. In the present study, 148.Fc microspheres pretreated with OSGE did not attach to CHO-P or CHO-E monolayers. This data is in accord with the effect of OSGE treatment on HL60 cell attachment to soluble P-selectin under flow (22), however, OSGE treatment does not affect flowing HL60 attachment to soluble E-selectin (22). Thus, either a region C-terminal to residue 148 on PSGL-1, that is spared with OSGE treatment of HL60 cells, or an OSGE-resistant ligand on HL60 cells mediates attachment to E-selectin under flow. Evidence from previous studies suggests that sLe^x is responsible for this OSGE-resistant E-selectin binding. Brunk et. al (45) have recently shown that sLe^x is sufficient to mediate inert microsphere attachment to and rolling on E-selectin under flow. In addition, HL60 cell sLe^x expression is not significantly altered by OSGE treatment (19) and O-linked saccharides carry most, if not all, sLe^x structures on PSGL-1 (24). Thus, our data taken together with previous studies suggest that: (1) a binding epitope on myeloid cells, which is not solely located within the N-terminal 148 amino acids of PSGL-1, is sufficient to mediate attachment to E-selectin; (2) this binding epitope is likely to be sLe^x; (3) OSGE-sensitive ligands are necessary to mediate HL60 cell sustained rolling on E-selectin; and (4) PSGL-1 which expresses high levels of sLe^x is likely to be such a ligand.

Our studies have two inherent caveats. First, we have used inert polystyrene microspheres coated with a single ligand, that is normally located on neutrophil microvilli, to study, in isolation, how a "reduced" neutrophil only bearing this ligand would behave. Certainly, the physical properties of a nondeformable microsphere differ greatly from that of a viscoelastic deformable neutrophil (46). Microspheres also lack many inherent

biological features of cells, such as membrane diffusion and receptor-cytoskeletal interactions (46). In addition, the behavior of an individual ligand, such as PSGL-1, on neutrophils is likely modified by interactions with other molecules located in proximity, such as L-selectin. Yet, the microspheres are a unique substrate for analyzing an individual adhesion molecule's function under flow conditions without the potential complications of "subtractive" methods. Moreover, the microspheres allow for the isolated investigation of the inherent capacity of the molecular structure of a given ligand to mediate adhesion without potential structural advantages that the ligand may have on a cell, i.e. deformability, proximity to other ligands.

Secondly, our experiments used PSGL-1 produced from COS and CHO cells co-transfected with HL60 cDNA and fucosyltransferase VII cDNA, and the extent to which the behavior of this PSGL-1 reflects that of native PSGL-1 expressed on leukocytes may be questioned. However, numerous studies have confirmed the validity of recombinant PSGL-1 as a model for the native glycoprotein. For instance, PL1 inhibits P-selectin binding to both human leukocytes (20) and to recombinant PSGL-1 which is expressed with a branching enzyme for O-linked glycans and an α1-3fucosyltransferase on CHO cells (43). Both native PSGL-1 (55) and PSGL-1 expressed in CHO cells (43) are sulfated on tyrosine and require this moiety for high affinity binding to P-selectin. Polyclonal antibody Rb3026 generated against PSGL-1 produced by COS cells blocks HL60 cell binding to CHO-P (23). Hence, there is ample precedent that studies with recombinant PSGL-1 provide valid insight into the function of native PSGL-1.

Based on static adhesion studies, Sako et al. (34) suggested that PSGL-1 functions via a recognition motif composed of the N-terminal anionic polypeptide segment and the sialylated O-linked oligosaccharide on Thr-16. The authors indicated that only the sialylated structure is required for E-selectin binding while both components of the motif

are required for high affinity binding to P-selectin (34). Our findings refine and extend this schema as it applies to flow conditions. The data demonstrates that PSGL-1 has two or more sites that are sufficient for E-selectin binding, the sLe^x-glycan on Thr-16, and one or more structures to the C-terminal of amino acid 19. Both sialylated structures and the N-terminal anionic polypeptide are required for PSGL-1 attachment to P-selectin, and sialylated structures are necessary for sustained rolling on P-selectin. Interestingly, for the 19.ek.Fc microspheres, the level of sLe^x on Thr-16 required for sustained stable rolling on P-selectin is greater than that necessary for attachment. In agreement with previous studies, our results suggest that the bonds between selectins and sialylated, fucosylated carbohydrate counter receptors, such as sLe^x, are especially suited to support rolling. Alon et. al (56) measured the off rate for the P-selectin - neutrophil counter receptor bond and attributed the ability of this bond to mediate rolling to its rapid kinetic and unique tensile properties. Mathematical models have emphasized the importance of similar features of the selectin - counter receptor bonds underlying neutrophil rolling (57, 58).

Given a constant selectin structure and presentation, the issue remains whether the unique kinetic and tensile properties of the selectin - PSGL-1 bond are, at least, partially a function of the inherent molecular structure of PSGL-1 or are exclusively dictated by other factors, such as the strategic presentation of PSGL-1 on the leukocyte. Indeed, PSGL-1 (20) and L-selectin (25) have been localized on neutrophil microvilli, the proposed initial contact points of a neutrophil with endothelium in vitro (59). According to this paradigm, the strategic location of PSGL-1 on flowing leukocytes affords the ligand with the necessary proximity to P- or E-selectin expressed on activated endothelium to potentially form bonds with these molecules. Other structural factors that may improve the opportunity for PSGL-1 to mediate attachment and rolling on P- or E-selectin include leukocyte deformability under shear stress and membrane diffusability (46). However, our results indicate that PSGL-1, immobilized on an inert nondeformable microsphere, can

mediate attachment and rolling on P- and E-selectin under shear stress. Thus, if PSGL-1 is in proximity to potentially bind P- or E-selectin, the molecular structure of the ligand may be a crucial determinant of whether bonds form and give rise to the phenomenom of leukocyte attachment and rolling.

Finally, this study suggests the feasibility of numerous in vitro and in vivo applications. We have established a model for studying the contribution of an individual leukocyte ligand - endothelial receptor(s) interaction to the adhesion cascade. In addition to studying the individual contribution of leukocyte ligands, the combined contribution of multiple ligands could be studied by coupling these ligands to the same microspheres. Furthermore with the demonstration that PSGL-1 is sufficient to selectively attach to activated human endothelium, potential diagnostic and therapeutic applications are conceivable. For instance, PSGL-1 could be coupled to liposomes containing Technetium-99 or therapeutic agents in order to image or deliver drugs to areas of inflammation or atherosclerosis.

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Figure Legends

Figure 1. For a leukocyte to extravasate from the vascular compartment during the inflammatory response, it must undergo a complex and multi-step adhesion cascade with the activated endothelium (1-4). The leukocyte initially attaches to the endothelium and then rolls along the surface. This part of the cascade is reversible. If the leukocyte continues along the adhesion cascade, it arrests and finally spreads and transmigrates. Many molecules on the leukocyte and endothelial surfaces are involved in this adhesion cascade. We focused on the contribution of the interaction of P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes with P and E-selectin.

Figure 2. The selectins share a structural motif. These surface molecules have a unique extracellular region composed of an amino-terminal lectin domain, an epidermal growth factor (EGF) - like domain and two (L-selectin), six (E-selectin) or nine (P-selectin) short consensus repeat (SCR) units (of about 60 amino acids each) homologous to domains found in complement binding proteins (7-9). The carboxyl terminal shaded and open rectangles represent the transmembrane domain and cytoplasmic tail, respectively.

Figure 3. Activated vascular endothelium expresses P- and E-selectin. P-selectin is constitutively expressed and stored in Weibel-Palade bodies in endothelial cells. Within minutes after exposure to thrombogenic and inflammatory mediators, P-selectin can be detected on the endothelium surface. E-selectin expression is limited to activated endothelium. E-selectin synthesis is stimulated by a variety of inflammatory mediators, including cytokines and bacterial endotoxin, with maximal expression on cultured human umbilical vein endothelial monolayers 4 - 6 hours after activation.

Figure 4. Schematic representations of PSGL-1.Fc chimeric constructs used in this study. (**A**) The PSGL-1.Fc constructs consist of an amino-terminal region of intact or mutated human recombinant mature PSGL-1 fused to the CH2-CH3 Fc region of human IgG_1. (**B**) The 148.Fc, 19.ek.Fc and ΔY.148.Fc constructs were used. Closed bars indicate PSGL-1 segments. Open bars indicate human Fc segments. Open bar with X in ΔY.148.Fc indicates an internal deletion of amino acids 5 through 11 within the N-terminal polypeptide region. Shaded bar indicates the enterokinase cleavage site. Y represents tyrosine. Vertical lines with open circles denote potential O-linked glycosylation sites and closed circle indicates O-linked site at threonine 16. Vertical lines with shaded rectangles indicate potential N-linked glycosylation sites. (Not drawn to scale.)

Figure 5. PSGL-1 was immobilized on polystyrene microspheres. Protein A, a cell wall constituent of *Staphylococcus aureus* that binds avidly to the Fc region of IgG, was adsorbed to the surface of microspheres at a concentration of 300 μg/ml. The microsphere - protein A complex was then incubated with a PSGL-1.Fc chimera or human IgG_1 to form PSGL-1 or IgG_1 microspheres, respectively.

Figure 6. A parallel plate flow chamber was used to study PSGL-1 adhesion to P- and E-selectin under flow conditions. The parallel plate chamber consists of two stainless steel plates separated by a silastic gasket. A glass slide coated with a monolayer composed of human umbilical vein endothelial cells (HUVECs) or Chinese hamster ovary cells stably transfected with P- (CHO-P) or E-selectin (CHO-E) was placed in the chamber, and the entire apparatus was mounted on an inverted microscope. Beads were drawn through the chamber and their interaction with the monolayer was recorded via live time videomicroscopy. Bead attachment rates were calculated as #attachments/(mm^2*min).

Figure 7. A PSGL-1.Fc chimera molecule can be coupled to protein A microspheres. 10 μm microspheres were pre-coated with protein A. These microspheres were then incubated with various concentrations of the 148.Fc chimera. The 148.Fc chimera bound to the microspheres was detected with a mAb to PSGL-1 (a-e), PSL-275, and an appropriate FITC labeled secondary antibody. Isotype matched control mAb to ICAM-1, Hu5/3, did not recognize the 148.Fc chimera (f).

Figure 8. 148.Fc microspheres attached and rolled on TNF-α activated HUVEC monolayers under flow. 148.Fc or IgG_1 microspheres were perfused across TNF-α (4 hour, 25 ng/ml) activated or unactivated HUVEC monolayers. 148.Fc microspheres attached and rolled on TNF-α activated HUVEC monolayers while IgG_1 microspheres did not attach to the TNF-α activated HUVEC monolayers. 148.Fc microsphere attachment was blocked by a mAb to E-selectin (7A9) but unaffected by an isotype matched control mAb to Class I (W6/32). 148.Fc microspheres did not attach to unactivated HUVEC monolayers. (Estimated shear stress = 2 dynes/cm^2; * $p < 0.05$; n=3).

Figure 9. 148.Fc microspheres rolled on TNF-α stimulated HUVEC monolayers. The image shows two 148.Fc microspheres (white spheres) rolling over a TNF-α activated HUVEC monolayer (gray background). Images were captured, every 0.6 seconds, from a videotape of the experiment and layered together to give the composite image shown. Note that the 148.Fc microspheres translated in the direction of the flow with a non constant velocity. The average velocity of 10 different 148.Fc microspheres was determined and found to be 14 μm/sec, which is < 3 % of the hydrodynamic velocity of a non-interacting hard sphere translating 50 nm from the surface (60). The length of the image shown is 100 μm. (Estimated shear stress = 2 dynes/cm^2).

Figure 10. 148.Fc microspheres attached to and rolled on CHO-P and CHO-E monolayers. 148.Fc microspheres attached and rolled on CHO-E and CHO-P monolayers while IgG_1 microspheres did not attach to either CHO monolayer. 148.Fc microsphere attachment to CHO-E and CHO-P monolayers was blocked by a mAb to E-selectin (7A9) and a mAb to P-selectin (HPDG2/3), respectively, but unaffected by control mAb (W6/32). 148.Fc microspheres did not attach to the parental CHO cell line (data not shown). (Estimated shear stress = 2 dynes/cm^2; * $p < 0.05$; n=3).

Figure 11. O-sialoglycoprotein endopeptidase (OSGE) abolishes 148.Fc microsphere attachment to CHO-P and CHO-E monolayers. (**A**) 148.Fc microspheres were incubated with buffer (top two panels) or 160 μg/ml OSGE (bottom two panels) at 37°C for 30 minutes. Untreated (a) or OSGE treated (b) 148.Fc microspheres were incubated with a mAb to PSGL-1 (PSL-275) (open histograms) or an isotype matched control mAb to ICAM-1 (Hu5/3) (shaded histograms) and subsequently detected using a FITC labeled secondary antibody. As a control for the specificity of OSGE, the presence of the human Fc region of the 148.Fc chimera was detected on the 148.Fc microspheres. Untreated (c) or OSGE treated (d) 148.Fc microspheres were incubated with a FITC labeled polyclonal antibody to human Fc (open histograms) or, control, mouse IgG (shaded histograms). Results shown are representative of n = 2-4 separate experiments. (**B**) Treatment of 148.Fc microspheres with OSGE prior to use in the *in vitro* flow assay abolished 148.Fc microsphere attachment to CHO- E and CHO-P monolayers. (Estimated shear stress = 2 dynes/cm^2; *$p < 0.05$; n=3).

Figure 12. The 19 N-terminal amino acids of PSGL-1 are sufficient for attachment to CHO-E or CHO-P monolayers, and PSGL-1 residues 5 through 11 are necessary for the 148 N-terminal amino acids to support attachment to CHO-P, but not CHO-E monolayers. (**A**) Microspheres coated with the 19.ek.Fc mutant (open bars), containing the first 19 N-

terminal amino acids of PSGL-1, attached to CHO-P and CHO-E monolayers at a rate similar to 148.Fc microspheres (dark bars). Microspheres coated with the ΔY.148.Fc mutant, which consists of the first 148 N-terminal amino acids of PSGL-1 with an internal deletion of the residues in positions 5 through 11, attached to CHO-E monolayers at a similar rate as 148.Fc microspheres but did not attach to CHO-P monolayers (cross hatched bars). (Estimated shear stress = 2 dynes/cm^2; * $p < 0.05$; n=2). (**B**) A mAb to PSGL-1, KPL1, which requires N-terminal amino acids 5 through 11 to recognize PSGL-1 (Table 1), did not affect attachment of 148.Fc microspheres to CHO-E monolayers but eliminated attachment to CHO-P monolayers. (Estimated shear stress = 2 dynes/cm^2; * $p < 0.05$; n=2) (**C**) mAb KPL1 eliminated attachment of 19.ek.Fc microspheres to both CHO-E and CHO-P monolayers. (Estimated shear stress = 2 dynes/cm^2; * $p < 0.05$; n=2)

Figure 13. Effect of neuraminidase treatment of 19.ek.Fc microsphere on adhesive interactions with CHO-E and CHO-P monolayers under flow. Microspheres were coated with 50 μg/ml 19.ek.Fc, treated with neuraminidase, and perfused over CHO-E (open bars) or CHO-P (filled bars) monolayers. (a) The rate of attachment to CHO-E monolayers was significantly diminished, if not eliminated, by treatment with neuraminidase. (b) In contrast, the rate of attachment to CHO-P monolayers was unaffected by neuraminidase treatment. (c) Accumulation of 19.ek.Fc microspheres on CHO-P monolayers was significantly reduced by treatment with neuraminidase. (Estimated shear stress = 2 dynes/cm^2, * $p < 0.05$; n=3).

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Figure 1

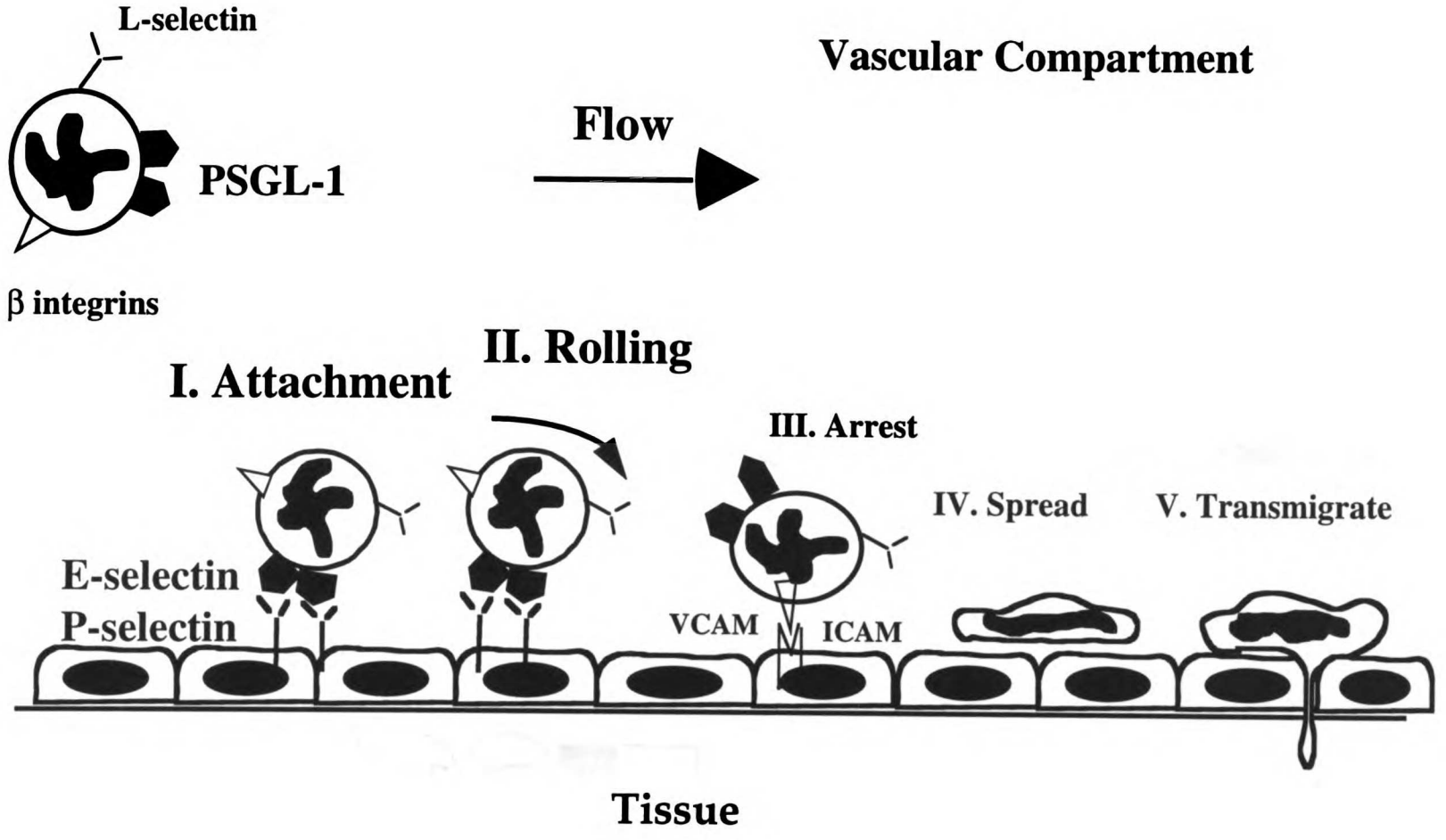
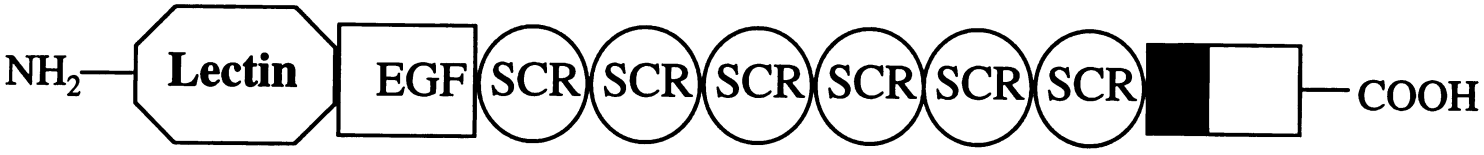
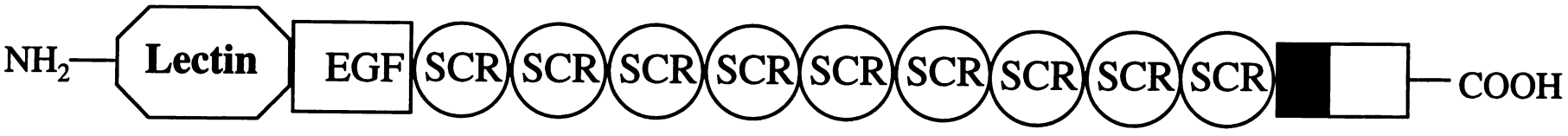

L-selectin
Vascular Compartment
Flow
PSGL-1
β integrins
I. Attachment
II. Rolling
III. Arrest
IV. Spread
V. Transmigrate
E-selectin
P-selectin
VCAM
ICAM
Tissue

Figure 2

E-selectin



P-selectin



L-selectin

Figure 3

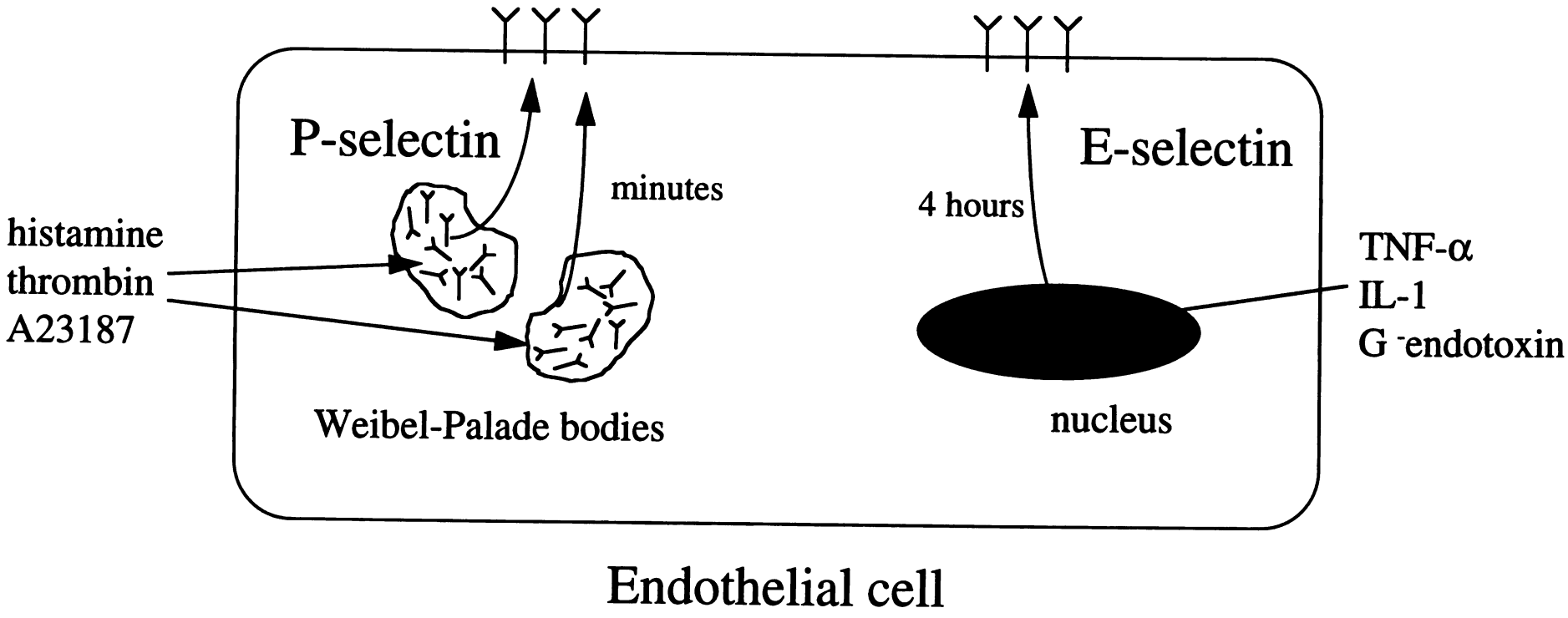

P-selectin
minutes
E-selectin
4 hours
histamine
thrombin
A23187
TNF-α
IL-1
G⁻endotoxin
Weibel-Palade bodies
nucleus
Endothelial cell

Figure 4A

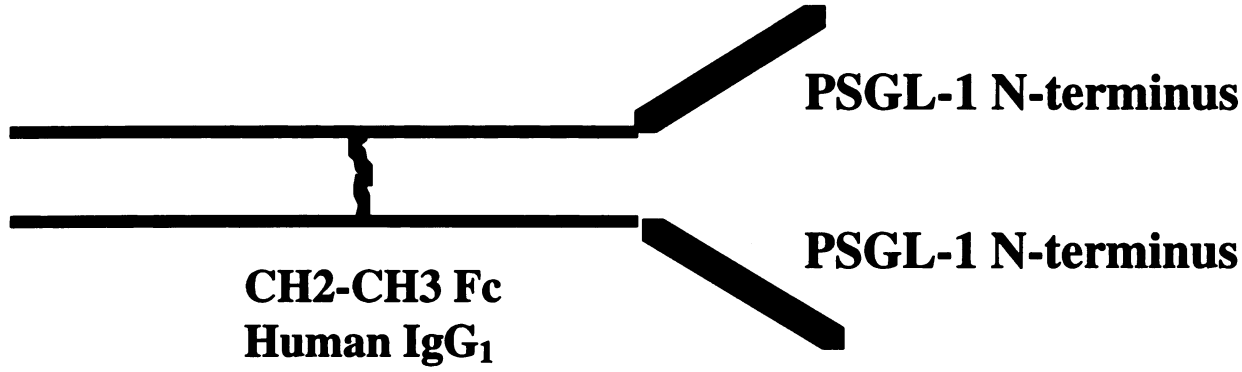


Figure 4B

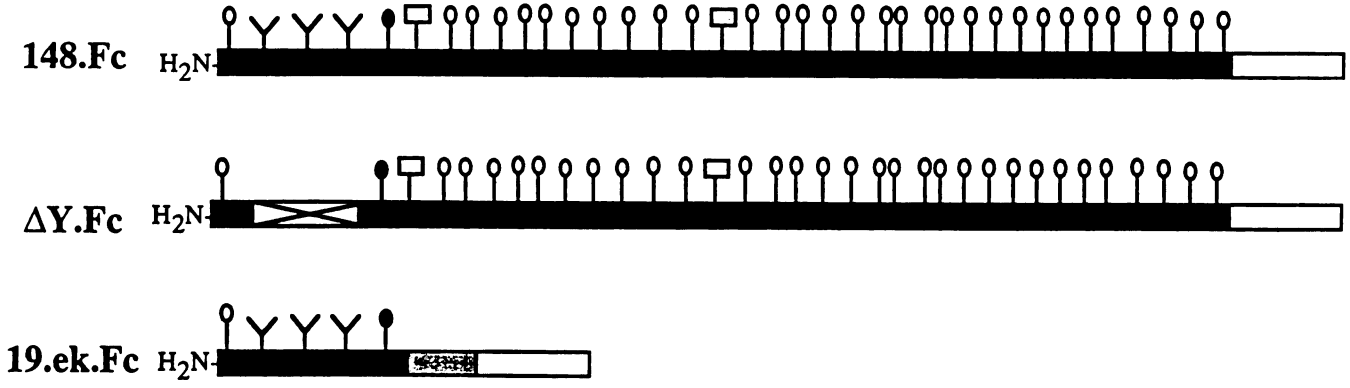


Figure 5

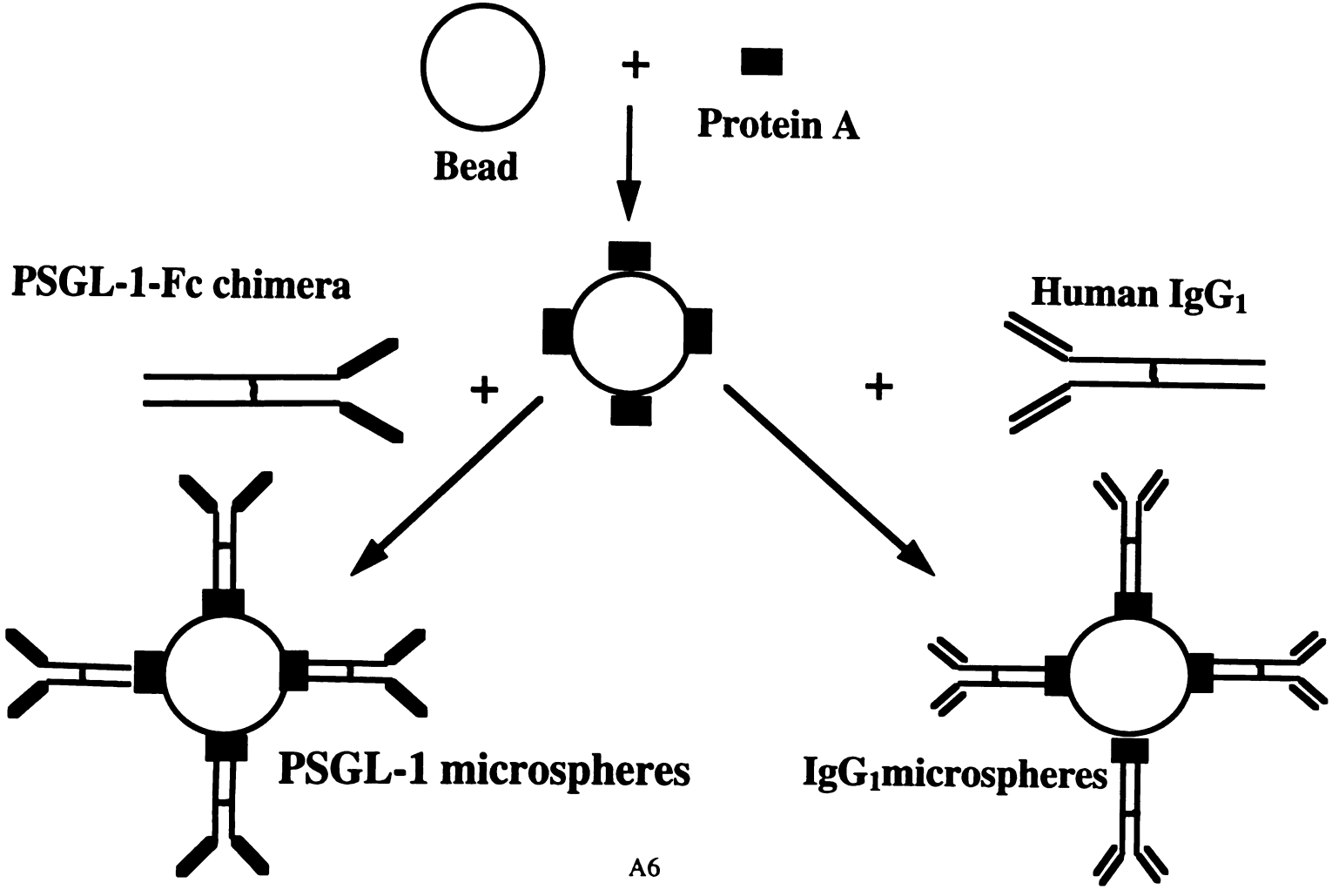
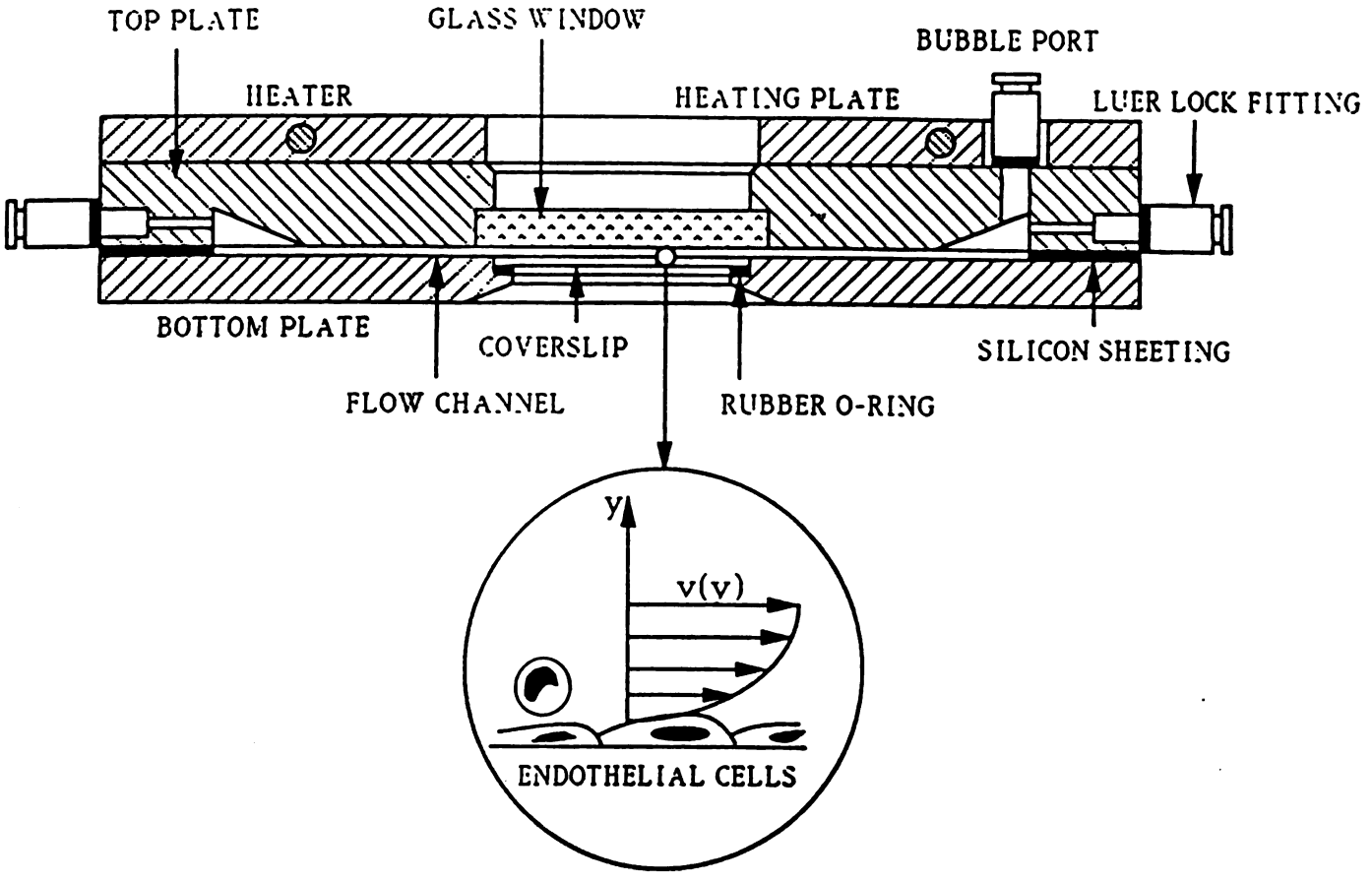

Bead
+
Protein A
PSGL-1-Fc chimera
+
Human IgG1
+
PSGL-1 microspheres
IgG1microspheres

Figure 6



Channel dimensions: 50.0 X 5.0 X 0.25 mm

- **Two dimensional fully developed laminar flow**
- **Shear stress levels between 0.2 - 8.8 dynes/cm²**

Figure 7

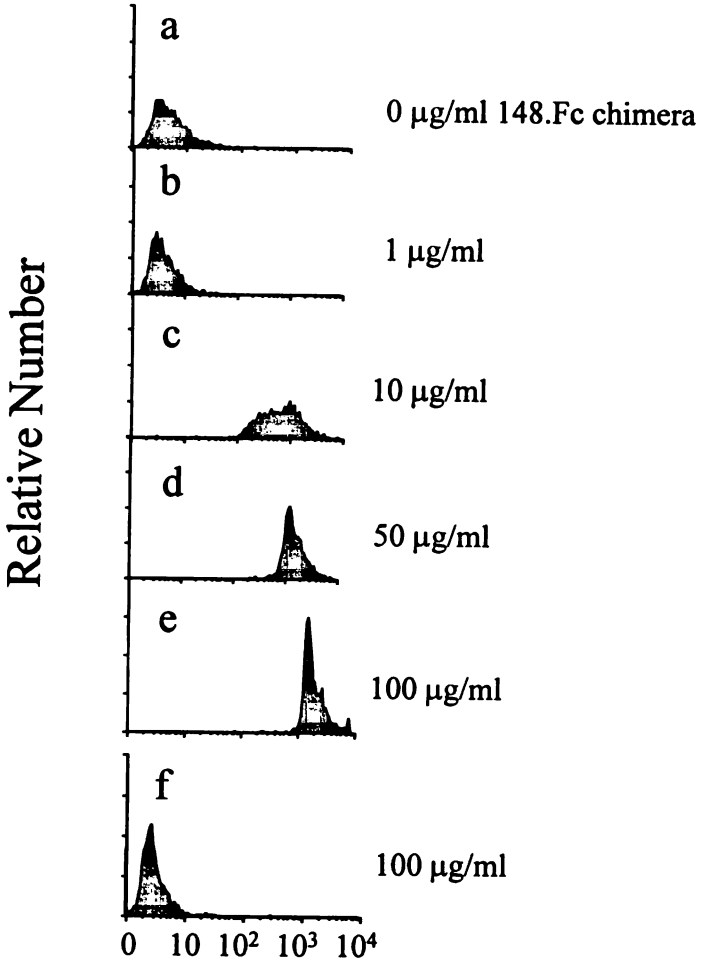

a
0 µg/ml 148.Fc chimera
b
1 µg/ml
c
10 µg/ml
d
50 µg/ml
e
100 µg/ml
f
100 µg/ml
Relative Number
0 10 10² 10³ 10⁴
Log Fluorescence

Figure 8

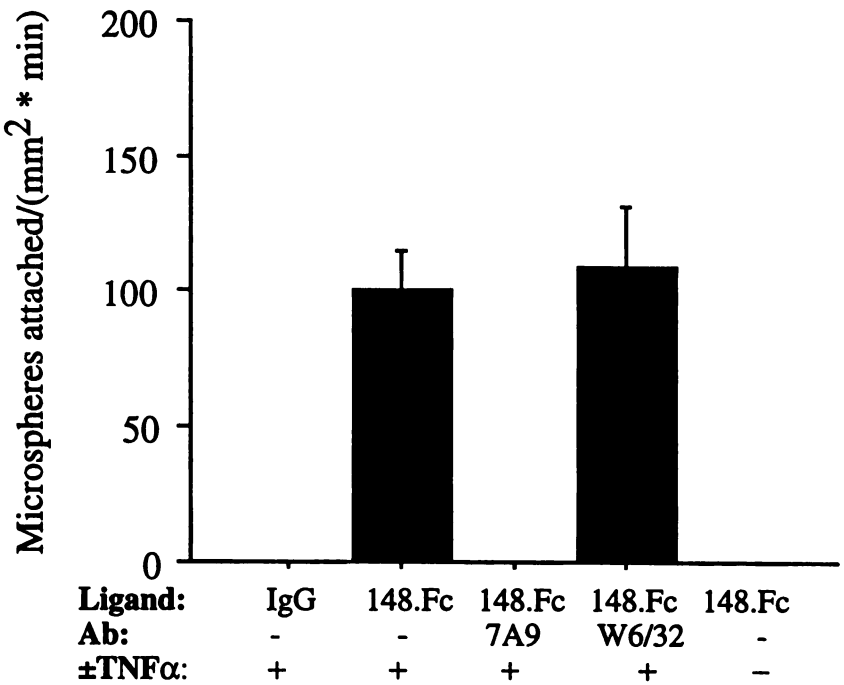
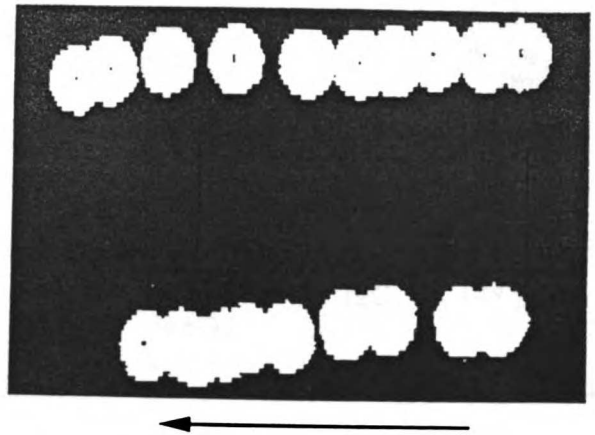

Microspheres attached/(mm2 * min)
200
150
100
50
0
Ligand: IgG 148.Fc 148.Fc 148.Fc 148.Fc
Ab: - - 7A9 W6/32 -
±TNFα: + + + + –

Figure 9



Direction of Flow

Figure 10

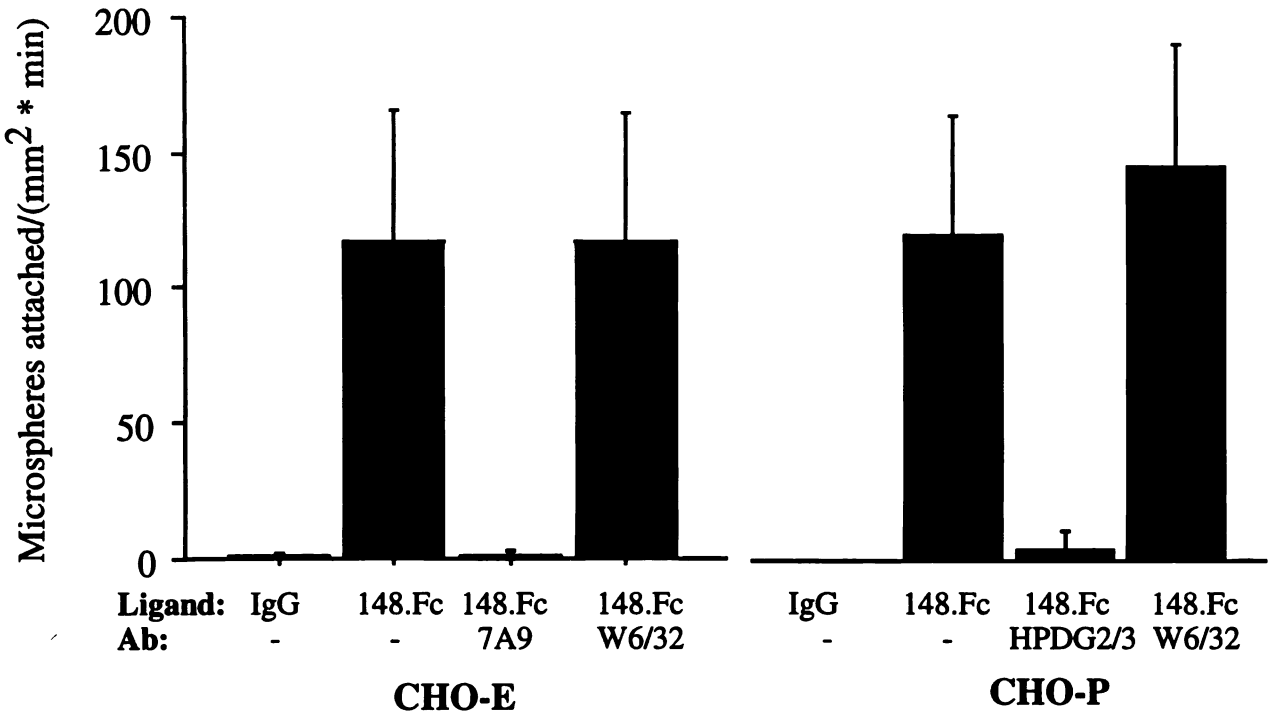

Microspheres attached/(mm2 * min)
200
150
100
50
0
Ligand: IgG 148.Fc 148.Fc 148.Fc IgG 148.Fc 148.Fc 148.Fc
Ab: - - 7A9 W6/32 - - HPDG2/3 W6/32
CHO-E
CHO-P

Figure 11A

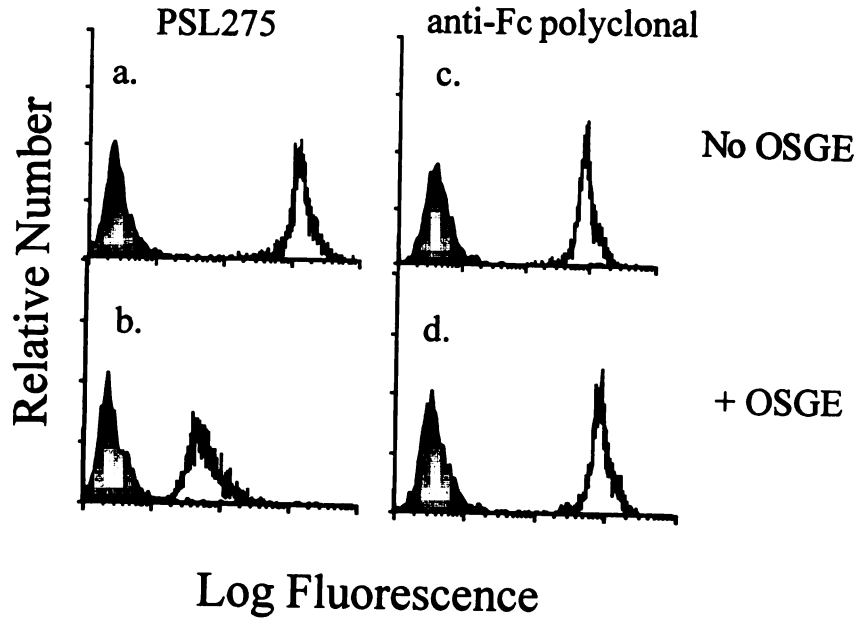

PSL275
anti-Fc polyclonal
a.
c.
No OSGE
b.
d.
+ OSGE
Relative Number
Log Fluorescence

Figure 11B

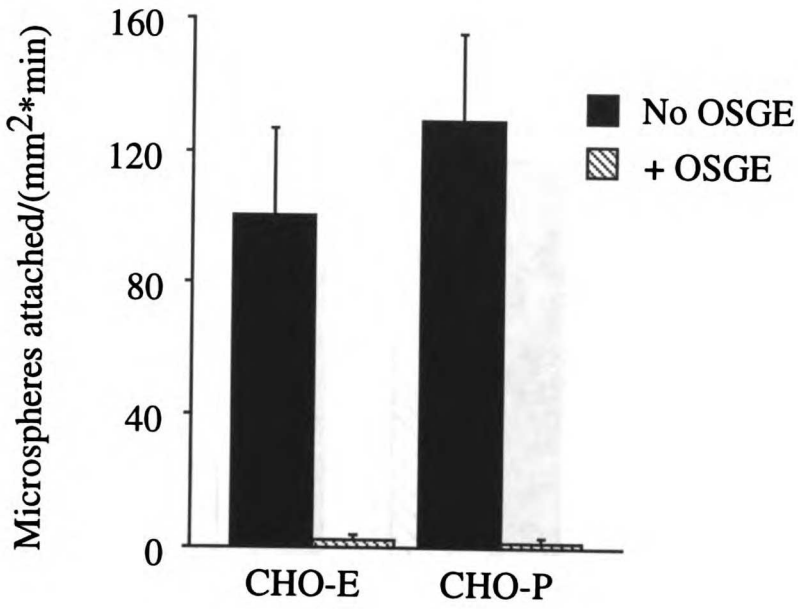

160
120
80
40
0
Microspheres attached/(mm2*min)
No OSGE
+ OSGE
CHO-E
CHO-P

Figure 12A

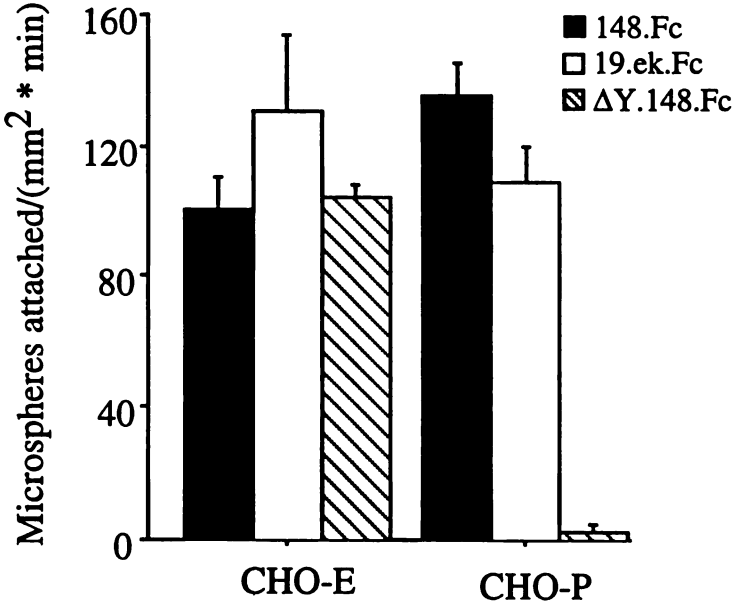

Microspheres attached/(mm2 * min)
160
120
80
40
0
148.Fc
19.ek.Fc
ΔY.148.Fc
CHO-E
CHO-P

Figure12B

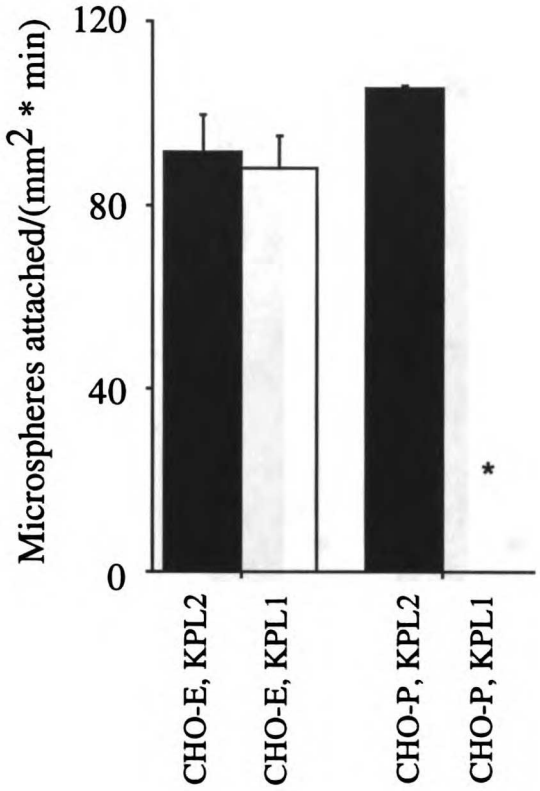

Microspheres attached/(mm2 * min)
120
80
40
0
*
CHO-E, KPL2
CHO-E, KPL1
CHO-P, KPL2
CHO-P, KPL1

Figure 12C

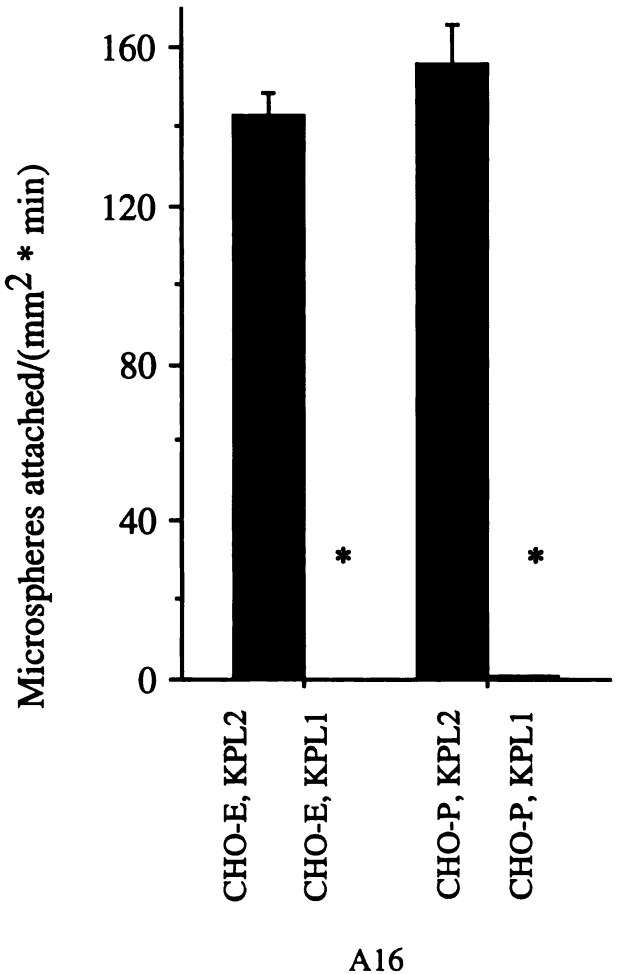

Microspheres attached/(mm2 * min)
160
120
80
40
0
*
*
CHO-E, KPL2
CHO-E, KPL1
CHO-P, KPL2
CHO-P, KPL1

Figure 13

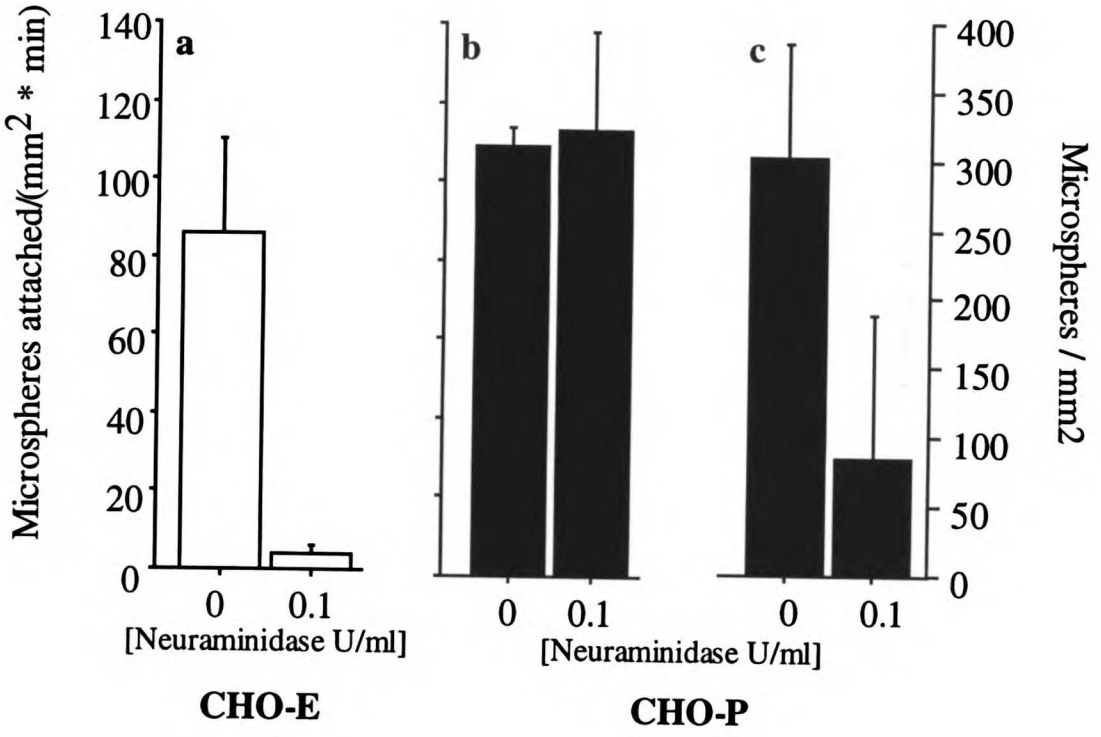

Microspheres attached/(mm2 * min)
140
120
100
80
60
40
20
0
a
0
0.1
[Neuraminidase U/ml]
CHO-E
b
c
400
350
300
250
200
150
100
50
0
Microspheres / mm2
0
0.1
0
0.1
[Neuraminidase U/ml]
CHO-P

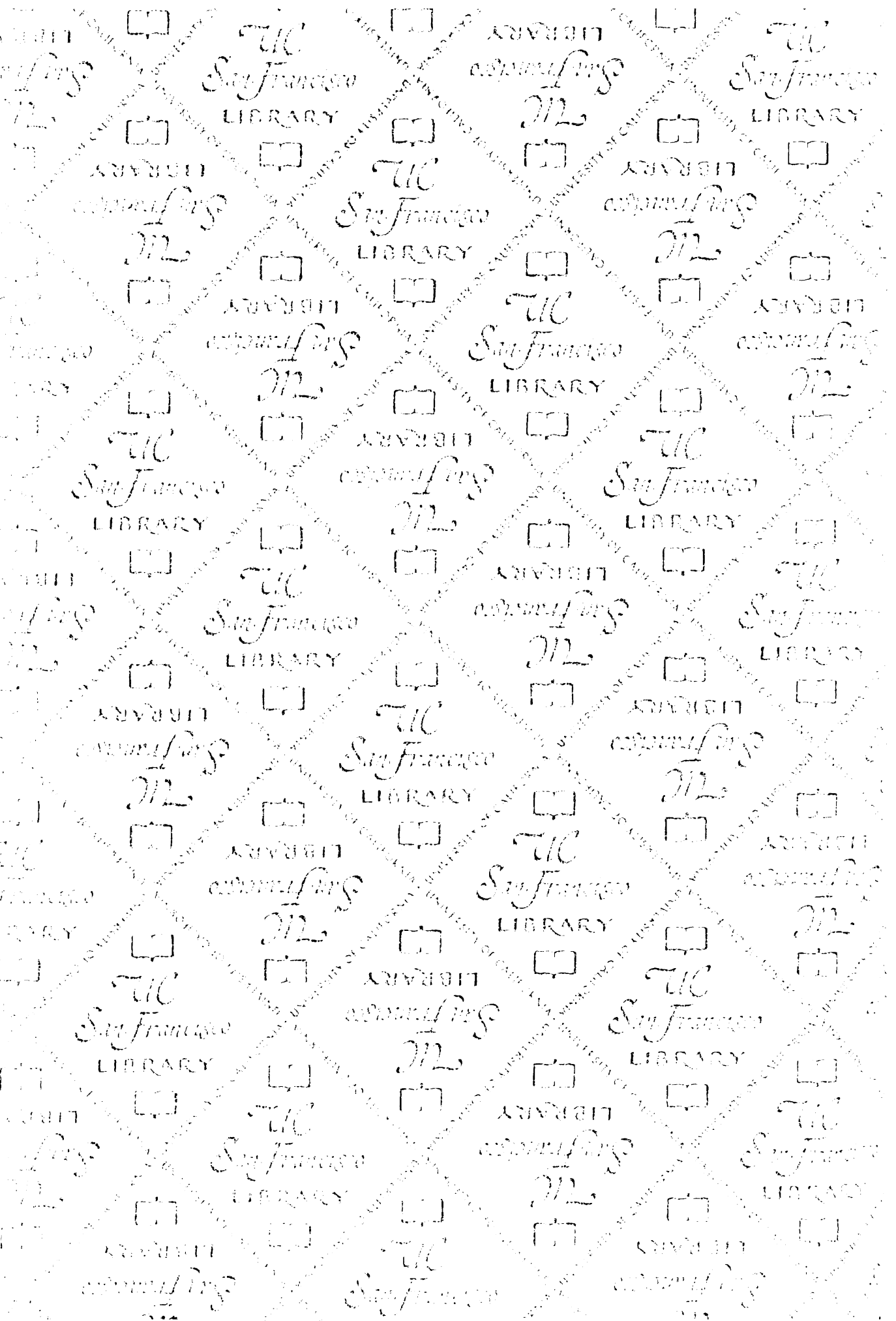
Table 1. Flow cytometric analyses of PSGL-1.Fc constructs on microspheres.

Mean Channel Fluorescence

mAb	IgG1	148.Fc	ΔY.Fc	19.ek.Fc
*NRS	243	60	83	167
Rb3026	137	554	520	335
*Hu5/3	16	12	27	42
KPL1	12	1595	11	461
KPL2	14	7	12	22

Microspheres were coated with indicated PSGL-1 molecules or human IgG and detected with various mAbs as detailed in methods.
Results typical of 2-4 separate experiments.

*Negative controls. NRS = Normal rabbit serum.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