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A high affinity human antibody antagonist of P-selectin mediated rolling

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Abstract

We have characterized the IgG form of a previously isolated and engineered single-chain Fv (scFv), named RR2r3s4-1, that binds to human PSGL-1. This fully human IgG was determined to have a K_d of 1.8 ± 0.7 nM by fluorescence quenching titration. It better inhibits P-selectin–PSGL-1 interactions than a commercially available murine monoclonal antibody KPL1 and better inhibits neutrophil rolling than KPL1. Thus, RR2r3s4-1 is the most effective antibody at inhibiting P-selectin–PSGL-1 interactions known. Specificity analysis reveals that RR2r3s4-1 does not cross react with murine PSGL-1 and thus requires more than tyrosine sulfate for binding to human PSGL-1. This evidence demonstrates the therapeutic potential of this antibody as a potent anti-inflammatory therapeutic.
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We have developed a high affinity, fully human IgG directed against the N-terminal 19 amino acids of P-selectin glycoprotein 1 (PSGL-1). PSGL-1 binds to P-selectin, a member of the selectin family of adhesion receptors which mediate rolling of leukocytes on the lumenal surfaces of the vascular endothelium [1]. Although many glycoproteins have been shown to support rolling *in vitro*, PSGL-1 is the major physiologically relevant ligand for P-selectin [2,3]. The N-terminal 19 amino acids of PSGL-1 have been shown to be both necessary and sufficient to mediate attachment and rolling on P-selectin *in vivo* [4]. Selectin mediated rolling is a particularly attractive target for therapeutic intervention because it occurs early in the inflammatory cascade before firm adhesion and extravasation into the surrounding tissue [5]. Blocking selectin binding may reduce tissue damage by both decreasing the number of leukocytes that enter the surrounding tissue and reducing the quantity of pro-inflammatory cytokines that are released. The search for high affinity antagonists of selectin adhesion as therapeutics for acute and chronic inflammation and ischemia–reperfusion injury states has been termed one of the Holy Grails of the selectin field [3].

Blocking P-selectin–PSGL-1 interactions has been shown to be therapeutically effective in several studies. A rat monoclonal antibody directed against the N-terminal 19 amino acids of mouse PSGL-1 inhibited neutrophil recruitment into chemically inflamed mouse peritoneum by $82\% \pm 7\%$ 2 h after stimulation and $59\% \pm 7.9\%$ 4 h after stimulation [6]. A recombinant PSGL-1-Ig chimera (rPSGL-1-Ig) consisting of the first 47 amino acids of the N-terminal portion of PSGL-1 fused to a human IgG_1 Fc has been generated as a therapeutic by Wyeth and has proven to be therapeutically effective in animal trials. rPSGL-1-Ig has been shown to accelerate thrombolysis and prevent reocclusion in a porcine model [7], to attenuate leukocyte–endothelial interactions after hemorrhagic shock in mice [8], and to protect against ischemia–reperfusion injury in cats [9].

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The disadvantage of rPSGL-1-Ig as a therapeutic is its low affinity which is equal to that of PSGL-1 for P-selectin ($K_d = 320 \pm 20$ nM) [10]. Another strategy to block P- selectin–PSGL-1 interactions is to improve the affinity of established monoclonal antibodies through various engineering techniques. High affinity therapies are advantageous because they can be used in lower dosages that both reduce the risk of harmful side effects and decrease cost. Although murine monoclonal antibodies against PSGL-1 such as KPL1, PL1, and PL2 are known [11,12], they are not suitable as therapeutics due to their immunogenicity. Only one human monoclonal against PSGL-1 exists in the literature and it does not have a published K_d [13].

Here, we characterize the binding properties of a high affinity IgG, named RR2r3s4-1, and a weaker affinity IgG parent, r2s4-2. Both of these IgGs were converted from single-chain Fvs (scFvs) that had been engineered to high affinity from a pool of PSGL-1 binders isolated from a yeast surface displayed non-immune library [14]. The K_ds were measured by tryptophan quenching titration and the antibody RR2r3s4-1 was verified to bind neutrophils by flow cytometric analysis. RR2r3s4-1 inhibited neutrophil rolling at a lower concentration than the murine monoclonal antibody KPL1. In the surface plasmon resonance (SPR) assays, murine PSGL-1 failed to inhibit RR2r3s4-1 binding to human PSGL-1, demonstrating specificity of this antibody for the human form. To our knowledge, RR2r3s4-1 is the most effective antibody at inhibiting P-selectin–PSGL-1 interactions known.

Materials and methods

IgG production and purification. COS-7 cells were maintained in 10% FBS/DMEM supplemented with 1 mM L-glutamine and 1% penicillin/streptomycin. Cells were transiently co-transfected with 25 μg of heavy chain and light chain using the liposomal-based reagent, TransIT (Mirus, Madison, WI), and cultured in serum-free media (Wyeth, Cambridge, MA) for 48 h. The supernatants were harvested, and the transfectants were maintained in serum-free media for another 72 h before the second harvest. IgGs were purified on a HiTrap rProtein A FF column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Following FPLC chromatography, samples were concentrated with Centriprep YM-10 (Millipore, Billerica, MA) and dialyzed into PBS.

Tryptophan fluorescence quenching titration. K_ds were determined in triplicate using an Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA). Between each measurement, the 3 ml quartz cuvette was thoroughly cleaned with 5 M HNO_3. Antibodies were diluted into PBS at a concentration of 20 nM. The ligand used was a monovalent form of 19.ek.Fc (a bivalent fusion of the N-terminal 19 amino acids of human PSGL-1 to a human Fc[4]) called 19.ek (which was a gift from Gray Shaw, Wyeth, Cambridge, MA) which lacks the Fc portion. A stock of 15 μM 19.ek was prepared and added stepwise 1 μl at a time to the diluted antibody in the cuvette to give points that differed by 5 nM in the ligand concentration. The excitation and emission wavelengths were 290 and 340 nm, respectively, and the excitation and emission slits were both set to 5 nm. The PMT voltage was set to high.

Inhibition ELISA. A 96-well ELISA plate (Corning, Acton, MA) was coated with 1 μg/ml human P-selectin (which was a gift from Diane Sako, Wyeth, Cambridge, MA) overnight at 4 °C in 10 mM MOPS, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.5. All dilutions and washes were performed with the coating buffer plus 0.05% Tween 20. The blocking buffer was the diluent/wash buffer plus 0.1% gelatin (Bio-Rad, Hercules, CA). The plates were blocked for 1 h. In a separate, U-bottom, polypropylene plate called dilution plate 1, a standard curve was made using a biotinylated form of 19.ek.Fc, called b.19.ek.Fc (a gift of Dr. Raymond T. Camphausen, formerly of Wyeth, Cambridge, MA), to determine the optimal concentration for inhibition. For the wells that were to receive blocking antibody, b.19.ek.Fc at 80 ng/ml (4× final concentration) was pre-complexed with a 1:500 dilution of streptavidin-HRP (Pierce, Rockford, IL) to yield 40 ng/ml (2× final concentration) of b.19.ek.Fc-strep-HRP and incubated at room temperature for 30 min.

In a separate dilution plate (dilution plate 2), blocking antibodies were started at a concentration of 1.5 μg/ml and diluted twofold. The KPL1 antibody was obtained from BD Pharmingen (San Diego, CA) and a negative isotype matched human IgG_1 control was generously provided by Wyeth (Cambridge, MA). After pre-complexing has been completed in dilution plate 1, 80 μl per well was transferred from dilution plate 1 to dilution plate 2 and incubated at room temperature for 15 min. After this incubation, 100 μl per well was transferred to the blocked P-selectin-coated plate and incubated at room temperature for 30 min. The plate was then washed and 100 μl of pre-warmed TMB (BioFx, Owings Mills, MD) was added to each well and incubated at room temperature for 10–15 min. 100 μl of 0.18 M H_2SO_4 was added to stop the reaction and the A_{450} was read on a Molecular Devices UVMax plate reader.

Fluorescence staining for flow cytometry. Neutrophils were isolated in the following manner. Whole blood was mixed 4–1 with 4.5% dextran in 0.9% NaCl. The mixture was left to settle for 90 min at room temperature. The top clarified fraction was collected and washed in sterile PBS. Red blood cells were lysed by adding 9 ml of water, letting the cells sit in the water for 1–2 min, then adding 1 ml of 10× Hanks' balanced salt solution. Cells were analyzed within 1–2 h of the completion of the neutrophil isolation.

Neutrophils were resuspended in PBS plus 0.1% BSA and labeled with either an isotype matched negative control antibody, provided by Wyeth (Cambridge, MA), or biotinylated RR2r3s4-1. Primary labeling was performed on ice for 30 min at a concentration of 10 μg/ml (66.7 nM). After washing, cells were labeled with streptavidin-R-phycoerythrin (strep-PE) (Molecular Probes, Eugene, OR) at a dilution of 1:200 for 30 min on ice. The cells were then washed and were suspended in 180 μl PBS plus 0.1% BSA plus 20 μl of 7-amino-actinomycin-D (7AAD) (BD Pharmingen, San Diego, CA) and incubated for 15 min on ice. Analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA).

Rolling inhibition assay. Polystyrene plates were adsorbed with 10 μg/ml of soluble recombinant P-selectin (R&D Systems, Minneapolis, MN) in PBS, supplemented with 10 mM bicarbonate, pH 9, at 4 °C for 16 h, washed with PBS, pH 9, blocked with PBS/2% human serum albumin (HSA) at 4 °C for 2 h, and washed with PBS, pH 9. Substrates were assembled on the lower wall in a parallel wall-flow chamber and mounted on an inverted phase-contrast microscope. Human neutrophils were isolated as previously described [15] and resuspended in Hanks' balanced salt solution supplemented with 10 mM Hepes, pH 7.4 (H/H) containing 2 mM Ca^{2+}, 0.5% BSA. Neutrophils were perfused through the flow chamber at 1×10^6 cells/ml at 0.3 dyne/cm^2 for 30 s and 1.0 dyne/cm^2 for 2 min. For the blocking assays, 1×10^6 neutrophils in 50 μl were first incubated with the various antibodies at 10, 2, 1, 0.5, and 0.1 μg/ml for 10 min at room temperature, and the volume was increased to 1 ml with H/H containing 2 mM Ca^{2+}, 0.5% BSA. Microscopic images of cells under flow were recorded on Hi-8 videotape using a Nikon plan 10×-objective microscope. Analysis was performed with a computerized imaging system consisting of a Pentium computer with MVC 150/40-VL boards (Imaging Technology, Bedford, MA). The isotype matched negative control antibody was provided by Wyeth (Cambridge, MA) and the PL2 antibody was obtained from Immunotech (Marseille, France).

Surface plasmon resonance competition assay. A Biacore 2000 instrument (BIAcore AB, Uppsala, Sweden) was used to analyze the

interaction between human anti-PSGL-1 antibodies and the biotinylated SGP-3 (b.SGP-3) peptide (a form of 19.ek that has been confirmed to be sulfated at the three tyrosines within the N-terminal 19 amino acids of PSGL-1 [16]). All experiments were performed at 25 °C using streptavidin-coated sensor chips (BIAcore) and HBS-P buffer (BIAcore; 20 mM Hepes [pH 7.4], 150 mM NaCl and 0.005% polysorbate 20 [v/v]) adjusted to 1 mM each $CaCl_2$ and $MgCl_2$. The streptavidin on the sensor surfaces was conditioned with three 1 min injections of a solution containing 1 M NaCl and 25 mM NaOH. The b.SGP-3 was allowed to bind to a SA sensor chip. Flow cell one was used as a reference surface.

The binding of human mAbs (50 nM) to the immobilized b.SGP-3 was competed with a murine PSGL-1 fusion to an Fc (m.PSGL-1.Fc) at concentrations of 100, 50, 25, 12.5, 1.6, and 0 times the antibody concentration. The human antibodies and competitors were premixed and allowed to equilibrate prior to injection. A positive control antibody that binds to tyrosine sulfate and a negative control antibody that does not bind to tyrosine sulfate alone were provided by Wyeth (Cambridge MA). Chips were regenerated with 5 µl of 0.1% TFA and equilibrated with running buffer. All curves were corrected for non-specific binding by an online baseline subtraction of ligand binding to a streptavidin surface in the control flow channel. Binding was analyzed using BIAevaluation software (V2.1; Pharmacia Biosensor, Uppsala, Sweden). The response was measured in resonance units (RU) representing the mass of bound mAbs at 20 s prior to the stop of the injection.

Results

Determination of dissociation constant (K_d)

The dissociation constant for r2s4-2 and RR2r3s4-1 was determined by tryptophan fluorescence quenching titration. The following equation was used to fit the data:

$$F = F_{\mathrm{E}} - \left[(E_{\mathrm{T}} + L_{\mathrm{T}} + K_{\mathrm{d}}) - \sqrt{(E_{\mathrm{T}} + L_{\mathrm{T}} + K_{\mathrm{d}})^2 - 4E_{\mathrm{T}}L_{\mathrm{T}}}\right] \times \frac{F_{\mathrm{E}} - F_{\mathrm{EL}}}{2E_{\mathrm{T}}} \qquad (1)$$

where F is the observed fluorescence, L_T is the ligand concentration, E_T is the total antibody concentration, F_E is the observed fluorescence without any ligand, and F_{EL} is the observed fluorescence intensity of the antibody-ligand complex at infinite ligand concentration [17].

Using this method, the K_d for r2s4-2 is 6.2 ± 3.0 nM and the K_d for RR2r3s4-1 is 1.8 ± 0.7 nM (examples of one curve fit for each clone are shown in Fig. 1). The K_d for r2s4-2 is about sevenfold lower and the K_d for RR2r3s4-1 is about 27-fold higher than those previously determined for the scFv forms of these antibodies [18]. It is possible that the constant regions of the IgG might orient the domains in the variable region slightly differently than they are oriented in an scFv format, thereby explaining the difference in K_ds. The single digit nanomolar affinity exhibited by RR2r3s4-1 for PSGL-1 is 2 orders of magnitude stronger than the affinity of P-selectin for PSGL-1 suggesting that RR2r3s4-1 should be effective at blocking neutrophil rolling in the low nanomolar range.

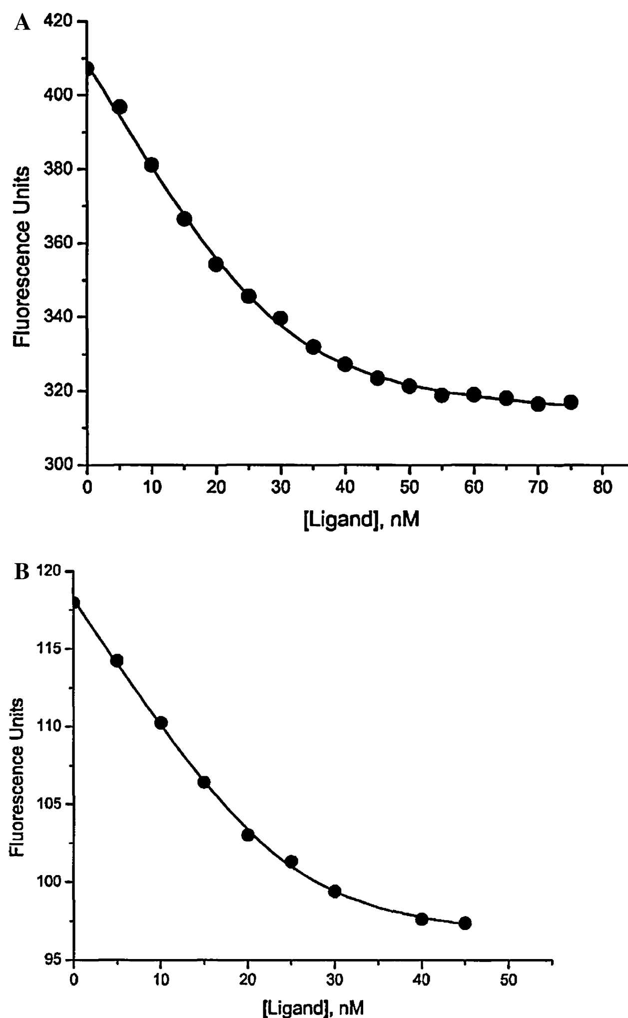

Fig. 1. Fluorescence quenching titrations. (A) Example of one titration of clone r2s4-2. (B) Example of one titration of clone RR2r3s4-1.

Binding of RR2r3s4-1 to human neutrophils

The antibody RR2r3s4-1 was raised against an artificial construct, b.19.ek.Fc, that contains only the terminal 19 amino acids of PSGL-1. It is important to demonstrate that this antibody binds to PSGL-1 expressed on the surface of human neutrophils. Fig. 2 shows that 67 nM of biotinylated RR2r3s4-1 binds to human neutrophils while a biotinylated, isotype matched negative control antibody does not.

Inhibition ELISA

An inhibition ELISA was used to compare the blocking effectiveness of the two antibodies presented in this paper with the previously characterized antibody KPL1. The basic setup of this experiment is shown in Fig. 3A. To determine the optimum concentration of b.19.ek.Fc for inhibition, a standard curve was made by serially diluting b.19.ek.Fc that has been pre-complexed with strep-HRP

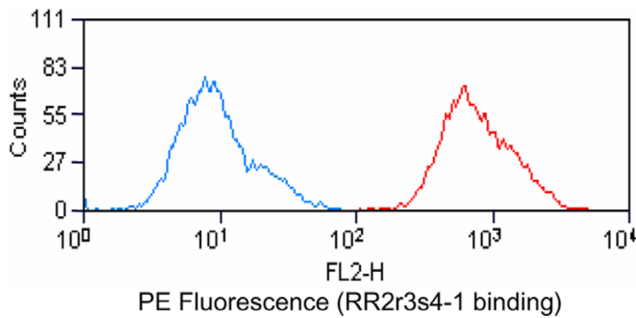

Fig. 2. Human neutrophils were stained with or 67 nM of biotinylated RR2r3s4-1 (red line) or 67 nM of a biotinylated, isotype matched monoclonal antibody (blue line) followed by streptavidin-PE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

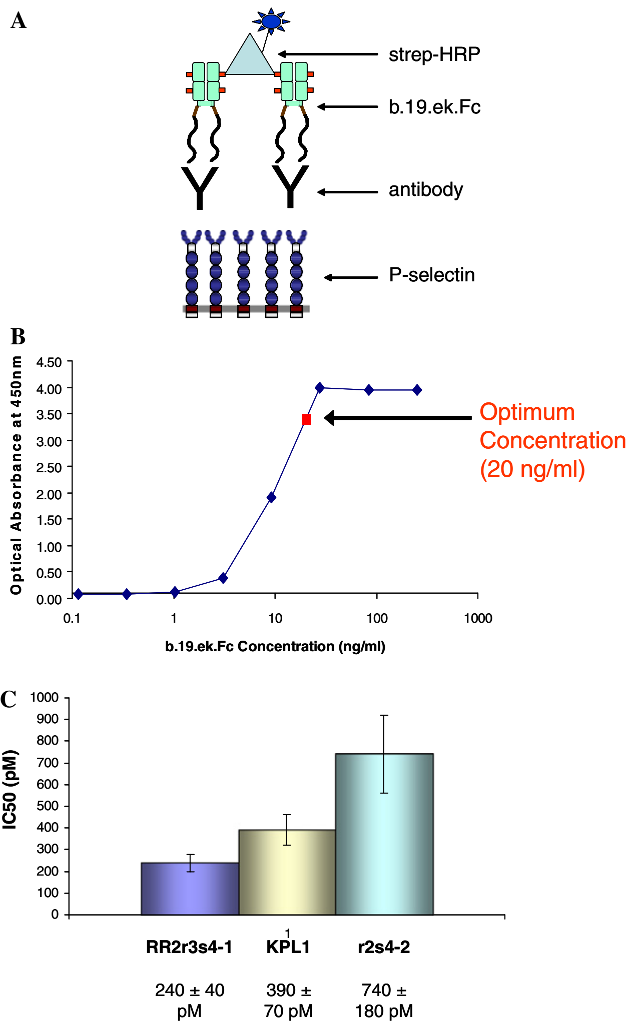

Fig. 3. Inhibition ELISA. (A) Schematic of the inhibition ELISA setup. Strep-HRP is pre-complexed with b.19.ek.Fc. This complex can be prevented from binding to a P-selectin-coated ELISA well by the presence of anti-PSGL-1 antibodies. (B) The optimal concentration for inhibition can be determined by making a standard curve for the binding of pre-complexed b.19.ek.Fc-strep-HRP to P-selectin-coated wells. (C) Bar graph showing the inhibition constants (IC_{50}) for RR2r3s4-1, KPL1, and r2s4-2.

(Fig. 3B). The inhibition constants can be determined by fitting the data to the following equation:

$$Y = B_{\max}\left(1 - \left(\frac{x^n}{K^n + x^n}\right)\right) + Y_2 \tag{2}$$

where Y is the absorbance at 450 nM, x is the inhibitor concentration, $B_{\max}$ is the maximum absorbance in the absence of inhibitor, K is the IC50, Y_2 is the absorbance at maximal inhibition, and n is a factor that allows for the adjustment of the slope of the curve at the value of the IC_{50}. RR2r3s4-1 has the lowest IC_{50}, and thus is a better inhibitor of P-selectin adhesion to PSGL-1 than KPL1 (Fig. 3C). As expected, r2s4-2 has the largest IC_{50} and therefore is the poorest inhibitor. It is important to mention that the actual value of the IC_{50}s in this experiment only have meanings within the context of this inhibition ELISA. They measure inhibition of a multivalent entity from binding to a multivalent surface and must not be confused with K_d values.

Rolling inhibition

The ability of various antibodies to inhibit neutrophil rolling is shown in Fig. 4. This *in vitro* rolling experiment was performed in a parallel plate flow chamber. Since it is possible for cells to form a transient tether, then detach and not roll it is necessary to define the difference between cells that form transient tethers and cells that actually roll. The definition we used for tethering and rolling is as follows: "transient tethers, cells that attached briefly to the substrate without rolling motions, and rolling tethers, cells that remained rolling on the substrate, i.e., moving at a mean velocity not more than a fourth the hydrodynamic velocity for at least 3 s after initial tethering" [19].

As Fig. 4 shows, RR2r3s4-1 has near 100% inhibition at a concentration of 3.3 nM while KPL1 has lost nearly half of its ability to inhibit neutrophil rolling at this concentration. The lower affinity antibody, r2s4-2, loses all of its ability to inhibit neutrophil rolling at 3.3 nM. The rolling

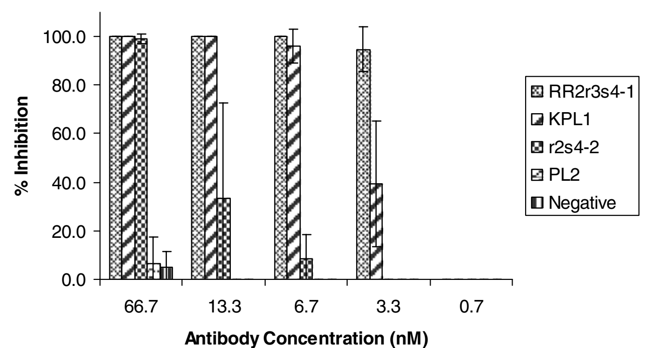

Fig. 4. Percent of neutrophils inhibited from rolling by various antibodies. The antibody PL2 is a mouse monoclonal antibody that binds PSGL-1 but does not bind in the site that is responsible for P-selectin binding. At the lowest concentration tested, 1.7 nM, none of the antibodies inhibited neutrophil rolling. The most potent rolling inhibitor was RR2r3s4-1 which blocked virtually 100% of cells rolling at a concentration of 3.3 nM.

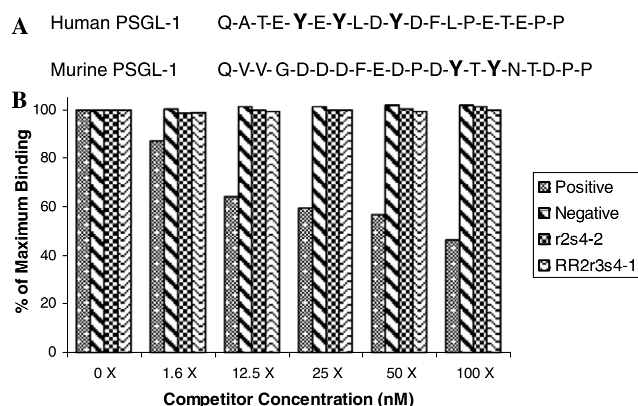

Fig. 5. Specificity of r2s4-2 and RR2r3s4-1 for human PSGL-1. (A) Amino acid sequence of the N-terminus of human and murine PSGL-1. The possible sites of tyrosine sulfation are shown in bold [2]. (B) SPR results indicating that neither r2s4-2 nor RR2r3s4-1 are inhibited from binding to human PSGL-1 in the presence of concentrations of murine PSGL-1 competitor in as high as 100 times the concentration of the antibody. For these experiments, human PSGL-1 (b.SGP-3) was coated onto a chip and pre-mixed antibody at 50 nM and murine PSGL-1 in the concentrations indicated were injected into the flow cell. The negative control is a known antibody that binds to human PSGL-1 and not murine PSGL-1 and the positive control is an antibody that binds to the tyrosine sulfate epitope.

inhibition data demonstrates that RR2r3s4-1 is the best antibody at inhibiting neutrophil rolling that has yet been characterized.

Specificity

The data presented in Fig. 5B indicate that both r2s4-2 and RR2r3s4-1 bind to human PSGL-1 even in the presence of concentrations of murine PSGL-1 that are 100 times the antibody concentration. The positive control antibody that is a known binder to the tyrosine sulfate epitope shows steadily decreased binding as the concentration of the murine PSGL-1 competitor increases. The ability to discriminate between murine and human PSGL-1 (whose amino acid sequences are compared in Fig. 5A) indicates that both r2s4-2 and RR2r3s4-1 should not exhibit adverse side effects from binding irrelevant tyrosine sulfate containing proteins.

Discussion

The paper demonstrates that a novel antibody, RR2r3s4-1, that has been isolated from a yeast surface displayed non-immune human library and engineered for high affinity binding to PSGL-1, has therapeutic potential. It binds PSGL-1 with single digit nanomolar affinity which is two orders of magnitude stronger than P-selectin binds PSGL-1. It both binds to the correct cell type and blocks these cells from rolling in an *in vitro* flow assay, indicating that it binds to native PSGL-1. Two assays, the rolling assay and an inhibition ELISA, demonstrate that this antibody inhibits binding of P-selectin to PSGL-1 better than the commercially available murine monoclonal KPL1 and therefore is the most effective antibody at inhibiting P-selectin–PSGL-1 interactions known. In addition, the SPR data provide evidence for the specificity of RR2r3s4-1. The next step is to perform a tissue cross reactivity assay in order to determine as more complete picture of the specificity of RR2r3s4-1. Hopefully, this antibody will become a major weapon in the ongoing fight against acute and chronic inflammatory conditions.

Acknowledgments

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