Isolation and Engineering of a High Affinity Antibody Against P-selectin Glycoprotein Ligand-1 (PSGL-1)

By
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Submitted to the Department of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of

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We aim to develop novel protein antagonists of P-selectin adhesion as anti-inflammatory therapeutics. Blocking P-selectin adhesion is particularly attractive because this adhesion mediates leukocyte rolling which occurs early in the inflammatory cascade before extensive tissue damage caused by the amplification of inflammation by pro-inflammatory cytokines. Currently, no subnanomolar antagonists of selectin adhesion are available. The low affinity of current antagonists results in the need for frequent administration and large doses in order to obtain inhibition. High affinity antagonists are desirable because they can be administered in smaller amounts thus reducing the risk of harmful side effects and reducing production costs.

Our approach for developing high affinity antagonists is to combine error prone PCR and *in vivo* homologous recombination to mimic in yeast the broad spectrum of mutagenic strategies exploited by B cells such as somatic hypermutation, receptor revision (V_H CDR replacement), receptor editing (chain shuffling), and amino acid insertions and deletions. Together with yeast surface display and flow cytometric screening (FACS), this approach has been used to effect at least a five order of magnitude affinity improvement in a single chain antibody (scFv) directed against the N-terminal 19 amino acids of P-selectin glycoprotein ligand-1 (PSGL-1).

Three rounds of engineering were performed after an initial pool of binders was isolated from a non-immune scFv library. Chain shuffling was found to be important for generating an improved mutant in the first round of engineering. For the final round of engineering, four different libraries were generated: one with random mutations, one with preferential replacement of the V_H CDR1, one with preferential replacement of the V_H CDR1 and the V_H CDR2, and one with preferential replacement of the light chain. All of these methods produced two order of magnitude affinity improvements except the light chain exchange library. However, the CDR exchange libraries gave equivalent affinity improvement despite the fact that they were 77 fold smaller than the random mutagenic library. In addition, an insertion in CDR2 of the V_H was isolated in the best binder from both of the CDR exchange libraries and this mutation could not have been found through random mutagenesis. These results suggest that chain shuffling is best used when the affinity of the antibody to be matured is weak ($> 1\ \mu M$). In addition, receptor revision is an equally robust method as random mutagensis for the generation of ultra-high affinity binders. The best antibody from the library with preferential replacement of V_H CDR1 and V_H CDR2 was converted to an IgG and characterized. It was found to better inhibit P-selectin binding to PSGL-1 than the commercially available antibody KPL1 in a static adhesion assay and an *in vitro* rolling assay. Our integrated approach, made possible by *in vivo* homologous recombination in yeast, decreases the likelihood of convergence upon

a single high affinity solution and increases the probability of obtaining an antibody with desired secretory properties and therapeutic potential. This facile method for combining all the mutational strategies used in nature should prove as a valuable tool in the antibody engineering field.

Thesis Supervisor: K. Dane Wittrup
Title: J.R. Mares Professor of Chemical Engineering, and Bioengineering

Dedicated to my parents, Gwen and Ronald, and to my brother Andrew, his wife Michele, and his son Alexander. Thanks for always inspiring me to do my best and supporting me unconditionally in the fulfillment of all my dreams.

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Chapter 1: Introduction

1.1 Technologies for Antibody Isolation and Engineering

Because molecular recognition is at the heart of biological processes, specificity and affinity are vital considerations for the development of therapeutic and diagnostic agents. Therapeutic applications require a specific interaction to be blocked and for the blocking agent to bind long enough to get a beneficial effect. Because we lack sufficient understanding of molecular interactions, *de novo* synthesis of binders is often difficult or impossible. Small molecules are the usual target of molecular design but, in general, they are not large enough to possess the level of interaction necessary for high affinity (subnanomolar) binding. In addition, their small size can lead to toxicity issues. Proteins are much larger entities with the potential for high specificity because of their large surface area and low toxicity because they are composed of the same building materials, amino acids, that are used within the human body. However, our understanding of protein folding and protein-protein interactions is not sufficient to rationally design novel protein binders.

Fortunately, evolution has provided an answer to the question of how to develop proteins with novel specificities. That answer is the antibody. Through their CDRs (complemetarity determining regions), antibodies can bind to virtually any antigen that exists. Their highly stable scaffold is compatible with CDRs of varying length and composition and the immune system has the ability to generate a large primary repertoire of $3.5 * 10^6$ if only combinatorial joining of V-J and V-D-J segments is considered[1]. Including other mechanisms of diversity generation such as nucleotide addition between

the V-J and V-D-J segments, the diversity of the human primary antibody repertoire is estimate to exceed 10^{10} [2]. In addition, the human immune system can further enhance antibody specificity and affinity in the periphery through somatic hypermutation, amino acid insertion and deletion[3-5], switching of light chains (receptor editing)[6-8], and exchange of portions of the variable region of the heavy chain (receptor revision)[9, 10].

The traditional methods of developing an antibody against a given target is to inject the target into an animal and either harvest the antibodies it creates or create monoclonal antibodies using hybridoma technology. Non-human antibodies present the problem that they will elicit an immune response when injected into a human. Because most therapeutic targets are human and a human cannot mount an immune response to a human protein, various methods have been generated to overcome this difficulty. To reduce the non-human content in an antibody, the entire variable region of a non-human antibody can be grafted onto a human constant region to generate a chimeric antibody. Furthermore, the non-human CDRs can be grafted onto a human antibody to create a humanized antibody.

Various methods have been developed to isolate fully human antibodies. Transgenic mice in which the mouse V-genes are replaced with human V-genes have been generated[11-13]. Although antibodies obtained from these mice can be useful without further manipulation, if their properties need to be modified they must be cloned into another system for further engineering. Technologies that do not require the need for immunization are available such as phage display[14, 15], yeast surface display[16, 17], and ribosome display[18, 19]. Phage display is the 'gold standard' for directed evolution of proteins because it has been around for the longest period of time[20]. Phage display

involves the fusion of a human antibody fragment to a phage surface protein, commonly pIII. There are usually one to three antibody fragments displayed per phage particle. Binding antibodies are found by panning the phage library against an antigen coated on a surface. Non-binders are washed away and the phage that bound are used to infect bacteria. The resulting phage can be harvested and further used for panning.

Yeast surface display involves the fusion of a single chain antibody (scFv) to the yeast surface mating receptor (Figure 1.1). There are thousands of antibody fragments displayed per cell in contrast to the one to three antibody fragments displayed per phage particle. Antigens coupled to fluorescent dyes can bind to the antibody fragments on the yeast surface thereby allowing for the detection and recovery of yeast cells encoding binders by using a fluorescence activated cell sorter, also know as FACS (Figure 1.2). Yeast display has many important advantages. First, it is a eukaryotic expression system and has foldases and chaperones making it more suitable for producing human proteins such as antibodies. In addition, the quantitative nature of the FACS screening process ensures that the antibodies are well expressed in yeast[21]. Poorly expressed clones will not be isolated because a minimum threshold fluorescence is necessary for a cell to be detected and sorted. Moreover, quantitative screens allow for fine affinity discrimination between clones that is not possible through panning.

1.2 A Non-immune Antibody Library in Yeast

Although antibodies have been engineered in yeast with great success[22], it was only recently that a yeast surface displayed, human, non-immune library was made available for the isolation of antibodies with novel binding specificities[17]. This library is approximately of $1.0 * 10^9$ diversity and was made from PCRing spleen and lymph node

mRNA using IgG and IgM heavy chain primers and λ and κ light chain primers (Figure 1.3). Binders were obtained against protein, peptide, and hapten antigens. This library has the advantage of being able to be propagated without an expression bias because the yeast promoter is tightly regulated. This promoter is galactose inducible and proteins that are deleterious to host growth can be maintained in the binding pool when the yeast are grown on glucose.

1.3 Yeast Surface Display: New Opportunities for Antibody Engineering

As previously described, yeast surface display has been proven as a method for antibody engineering and isolation. However, the flexibility provided by the yeast host can serve as a basis for advancing the way in which antibodies are engineered for higher affinity binding. For example, antibody engineering is usually performed in an iterative manner in which a method for diversity generation, such as random mutagenesis, CDR saturation mutagenesis, gene shuffling, or a combination of these methods is used followed by selection and repeated until an antibody of a desired affinity is isolated. However, this method does not exactly mimic how antibodies are affinity matured *in vivo*. Random mutagenesis does emulate somatic hypermutation but other *in vivo* methods are not readily used for antibody engineering. In the periphery, light chain shuffling, amino acid insertions and deletions, and receptor revision occur to further diversify antibodies. Although previous attempts have been made to mimic receptor editing and receptor revision using phage display, yeast greatly simplifies the way in which these methods can be used. The key simplification is the ease of which yeast recombine single stranded DNA that have stretches of homology. Those who work in phage must use more laborious methods, such as site specific recombination, which are

time consuming and limit diversity by confining crossover sites to specific regions. Utilizing yeast homologous recombination allows for one to easily mimic affinity maturation as it actually occurs in the human periphery and this simplification is the basis for Chapters 2 and 3 of this thesis.

1.4 Yeast Surface Display for the Isolation and Engineering of an Antagonist of P-selectin Adhesion to PSGL-1

In order to demonstrate the powerful new techniques for antibody engineering made possible through yeast surface display, we have chosen to isolate and engineer, in a way never previously attempted, a fully human antibody directed against P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is an important therapeutic target because it is the only physiologically relevant ligand for P-selectin known[23]. P-selectin binding to PSGL-1 is the first step in the process of how leukocytes migrate out of blood vessels into the surrounding tissue during an inflammatory response. The N-terminal nineteen amino acids of PSGL-1, which contain three potential sites of tyrosine sulfation and one O-linked site of glycosylation, have been shown to be necessary and sufficient for mediating adhesion with P-selectin *in vivo*[24]. The O-linked glycosylation contains sialyl Lewis X (s-Le^x) which is a tetrasaccharide that has been shown to bind P-selectin with a very weak affinity (K_d = 7.8mM)[25]. P-selectin, however, binds PSGL-1 with a K_d of 320 ± 20 nM[26]. As the crystal structure reveals, the increased binding affinity of P-selectin for PSGL-1 can be explained by the fact that at least two of the PSGL-1 sites of tyrosine sulfation are important for binding in addition to the s-Le^x [27]. Interestingly, PSGL-1 is a minor glycoprotein on myloid cells representing less than 1% of the surface s-Le^x however it is vital for P-selectin specific binding[28]. This fact makes PSGL-1 a more attractive target than s-Le^x because a PSGL-1 blocker could be dosed in lower quantities.

In addition, s-Le^x is involved in other functions such as leukocyte homing, thus, binding to PSGL-1 may be less likely to produce harmful side effects.

Upon exposure to certain pro-inflammatory mediators such as histamine, P-selectin is expressed on the endothelial cell surface within minutes. A drawing of the rolling along the endothelium mediated by P-selectin/PSGL-1 interactions is shown in Figure 1.4. This rolling is believed to activate the neutrophil so that it can firmly adhere to the endothelial cell surface. Firm adhesion is mediated by a different class of proteins known as integrins (Figure 1.5). After firm adhesion, the cell extravasates into the surrounding tissue. Each step in inflammation is accompanied by a release of cytokines that further amplifies the response. Blocking P-selectin adhesion is particularly attractive because leukocyte rolling occurs early in the inflammatory cascade before extensive tissue damage caused by the amplification of inflammation by pro-inflammatory cytokines.

A high affinity antagonists of P-selectin adhesion has the potential to have a major impact on the treatment of both acute and chronic inflammation. Treatment of diseases such as inflammatory bowel disease, ischemia/reperfusion injury, hemorrhagic shock, restenosis subsequent to angioplasty, asthma, and arthritis could be greatly advanced by such an intervention. Steroids, the major drug used to fight inflammation, are beneficial for reducing symptoms but can produce harsh side effects that are as intolerable for the patient as the illness being treated. High affinity antagonists may reduce inflammation at a dosage below which intolerable side effects are evident. The search for high affinity selectin inhibitors as anti-inflammatory therapies is so important that noted glycobiologist Ajit Varki of the University of California, San Diego has termed their development as one of the "Holy Grails" of the selectin field[23].

1.5 Thesis Overview

The second chapter of this thesis focuses on the development of a technique for utilizing homologous recombination in yeast for the creation of large, shuffled antibody libraries. The third chapter describes the isolation of a pool of novel scFvs that bind to PSGL-1. This chapter next explains how this pool of binders was used to create a single scFv and then describes how this scFv was affinity matured using a variety of techniques that mimic affinity maturation in the periphery of the human immune system. The fourth chapter describes the conversion of one of the highest affinity scFvs developed to an IgG and then shows that this antibody is more effective at blocking leukocyte rolling than a commercially available mouse monoclonal antibody known as KPL1.

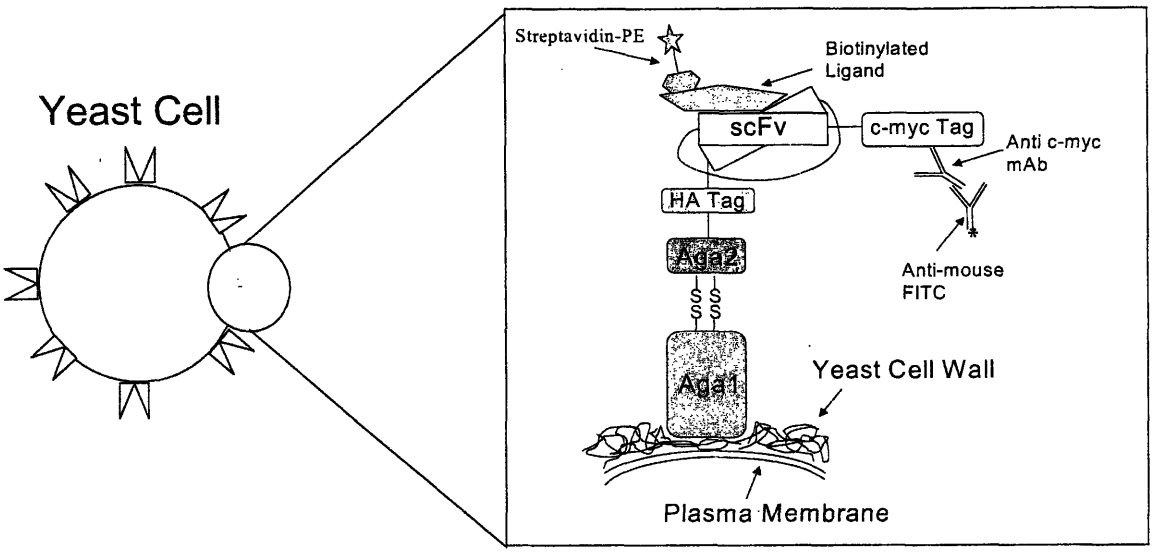


Figure 1.1: Schematic representation of an scFv displayed on the yeast's surface. The scFv is flanked by two epitope tags, an HA tag 5' to the scFv and a c-myc tag 3' to the scFv. The c-myc tag allows for normalization for protein expression. A bound biotinylated ligand can be detected by using fluorescently tagged streptavidin, such as streptavidin-phycoerythrin (PE).

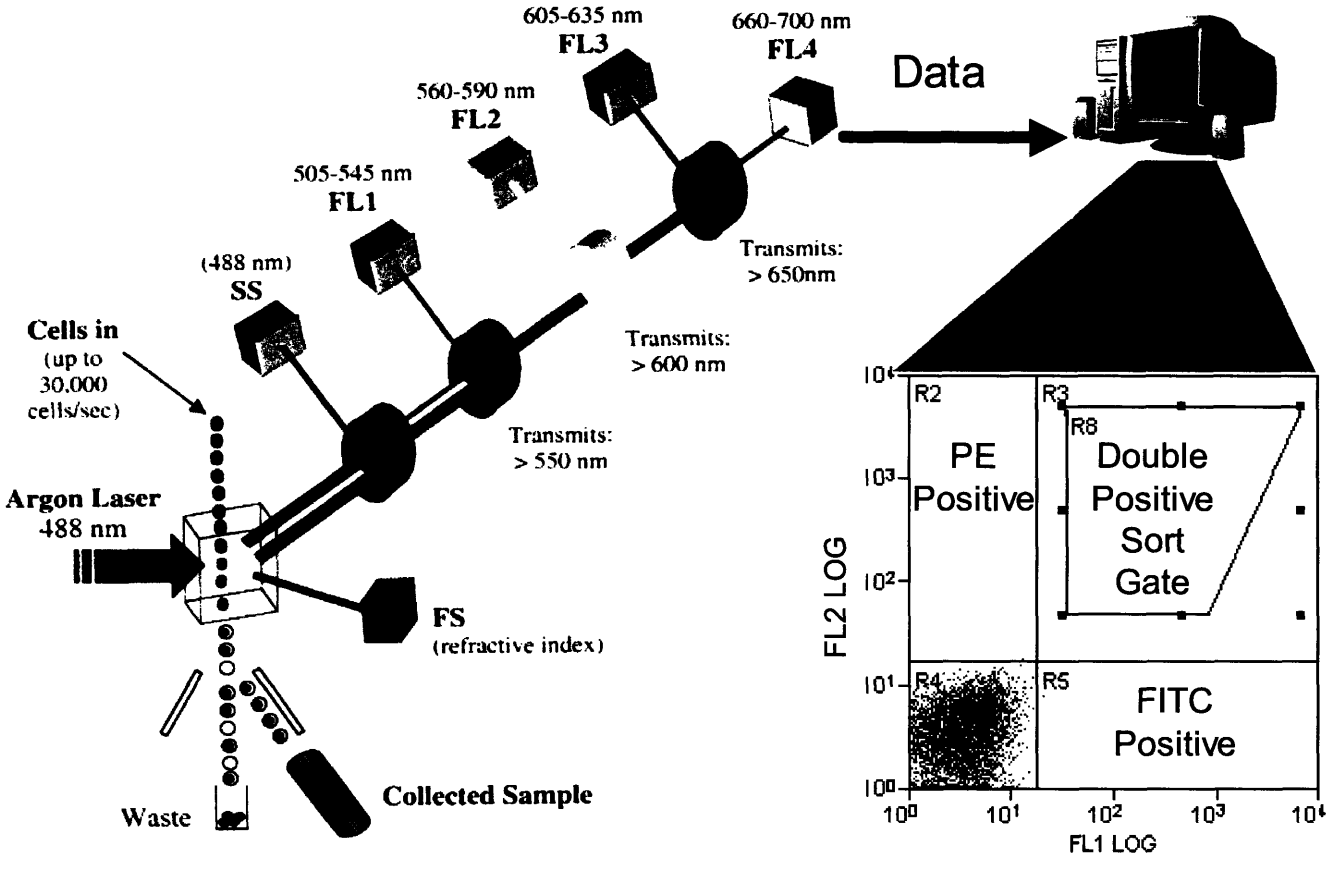


Figure 1.2: Schematic representation of a fluorescence activated cell sorter (FACS). Cells pass by an argon laser and the fluorophores bound to the cell surface are excited. The emission is detected by the appropriate detector and can be displayed on a computer in various formats. The bivariate histogram shows the FL2 signal, which corresponds to PE fluorescence, plotted against the FL1 signal, which corresponds to fluorescein isothiocyanate (FITC) fluorescence. The desired cells can be sorted by drawing a gate on the bivariate histogram. This figure is adapted from Graff (2002)[29].

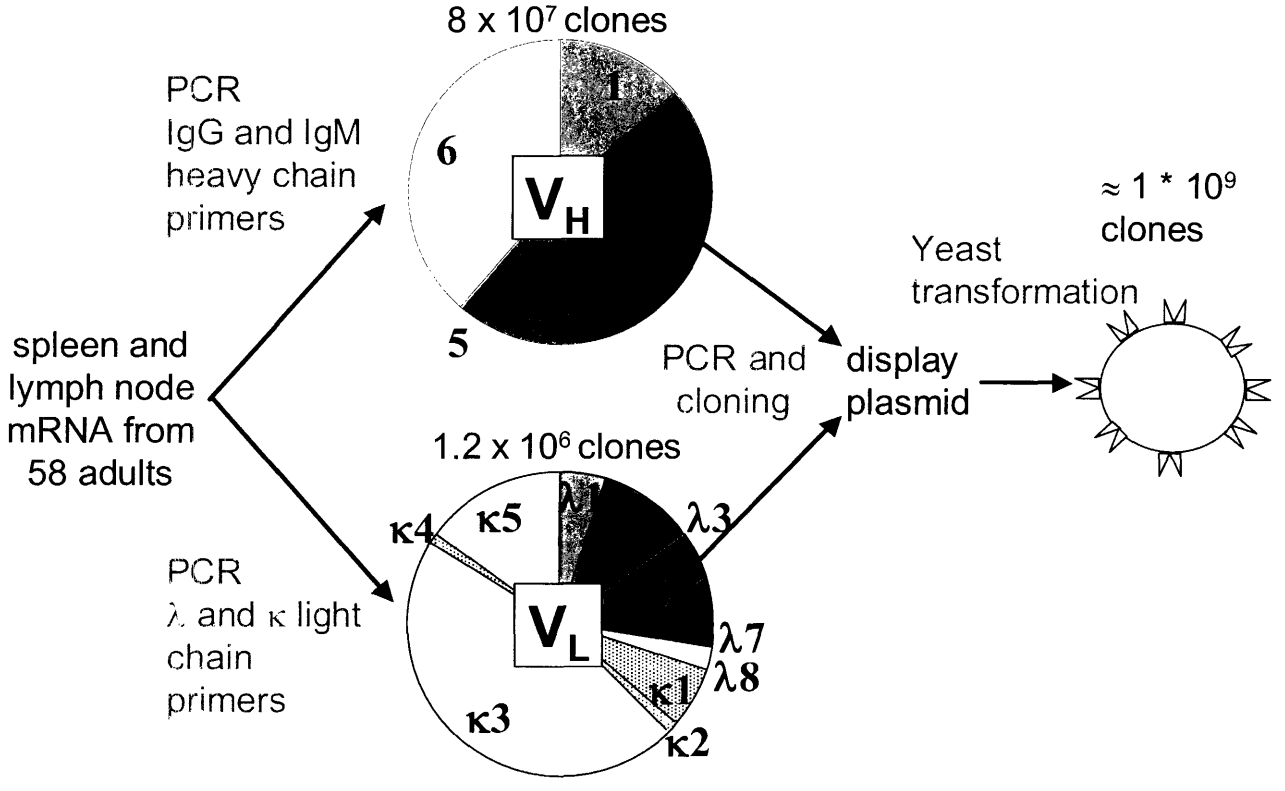


Figure 1.3: Schematic representation of the yeast surface displayed non-immune library's construction and chain diversity[17].

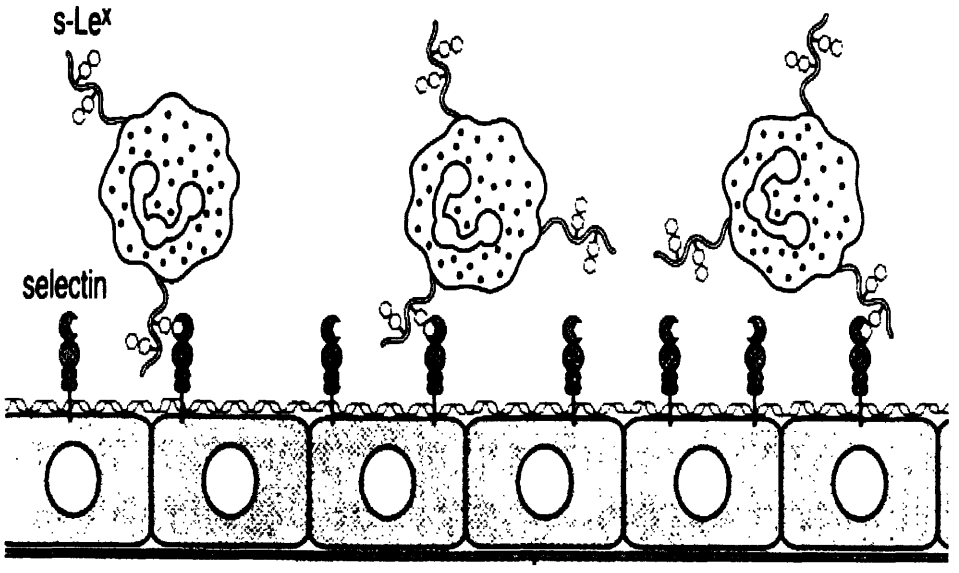


Figure 1.4: Diagram of selectin mediated rolling of leukocytes along the vascular endothelium. The leukocytes are shown with glycoproteins containing the sugar sialyl Lewis X (s-Le^x) of which PSGL-1 is one example. This picture is taken from Janeway (1999)[1].

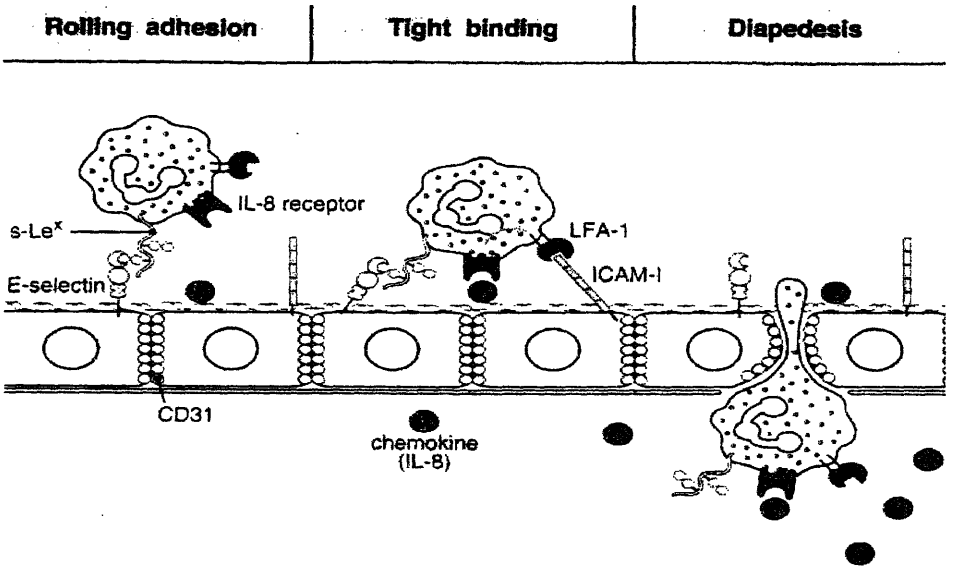


Figure 1.5: Leukocyte rolling is followed by firm adhesion of the leukocyte to the endothelium. In this picture E-selectin is shown however P-selectin also mediates rolling. LFA-1 is an integrin that binds to ICAM-1 allowing for firm adhesion of the leukocyte to the endothelium. Adhesion is followed by extravasation into the surrounding tissue. This picture is taken from Janeway (1999)[1].

Chapter 2: Shuffled Antibody Libraries Created by *In Vivo* Homologous Recombination and Yeast Surface Display*

2.1 Introduction

Various methods have been developed for the creation of diversity within protein libraries including random mutagenesis [30-32], *in vitro* DNA shuffling [33, 34], and site-specific recombination [35-38]. Random mutagenesis techniques utilize either a non-proof reading DNA polymerase in the presence of $MnCl_2$ [39], mutator *Escherichia coli* strains [16], or nucleotide analogues that cannot be correctly read by the DNA polymerase [40]. Random mutagenesis has the advantage of allowing for the isolation of beneficial mutations anywhere within the gene that may not be obvious *a priori*. However, point mutation methods do not allow for a radical restructuring of the contact regions and therefore are restrictive in the sequence space that can be probed. DNA shuffling methods have the advantage of being able to generate hybrid genes that contain portions of sequence space that have already proven to be functional. DNA shuffling consists of four steps: a DNase I digestion of the genes to be recombined, a PCR reassembly without primers, an amplification of recombined gene products of the correct size from the primerless PCR pool, and a ligation of the reassembled product into an acceptor vector. Although DNA shuffling has proven to be a highly effective method, numerous PCR and associated purification steps are required. Site specific recombination has the advantage of utilizing portions of known sequence space that have already proven functional, but it is often an impractical method because it requires the engineering of restriction enzyme

*This chapter is based upon Swers, J.S., Kellogg, B.A. & Wittrup, K.D. Shuffled antibody libraries created by *in vivo* homologous recombination and yeast surface display. *Nucleic Acids Res* **32**, e36 (2004).

sites into the genes to be combined. Often finding unique sites is difficult and the process can become tedious when multiple chimeric products are desired. Other forms of site specific recombination have been used to make libraries such as the n-CoDer antibody library in which CDRs were shuffled using specific primers and PCR reassembly [41], chain shuffled scFv libraries in which the shuffling was performed by PCR utilizing homology in the linker region between the heavy and light chains [42], and chain shuffled antibody libraries using Cre catalyzed recombination of antibody heavy and light chains that are flanked by *lox* sites [37, 38]. In all of these libraries the points in which gene exchange occur are fixed and prior knowledge of the gene sequences for the creation of PCR primer sets or engineering of specific sites is required.

A simple method for creating hybrid genes that does not require the extensive PCR steps of DNA shuffling, the pre-engineering of site-specific recombination methods, or ligation would be desirable. In this paper, we present a technique for creating large, chimeric antibody libraries using plasmid reconstruction by homologous recombination in the yeast *Saccharomyces cerevisiae* [43, 44]. This method allows for the coupling of diversity generation and protein production within the host organism rather than having a separate *in vitro* diversity generation step. *In vivo* recombination in yeast has been used in the past to create hybrid genes [45-47] and has been successfully used to generate a library for the directed evolution of a heme peroxidase enzyme [47]. However, this technique has not been exploited for the generation of antibody libraries. This absence can be explained by the fact that most *in vitro* antibody engineering is performed using phage which relies upon the machinery of *E. coli* for gene propagation and protein production. Although intra (Figure 2.1A) and inter-molecular recombination (Figures 2.1B and 2.1C)

have both been performed in *E. coli* to make hybrid genes, inter-molecular recombination has generally been used for plasmid construction and not recombination [48]. Inter-molecular recombination is inherently more flexible than intra-molecular recombination because only one (Figures 2.1B and 2.1C) or none (Figure 2.1D) of the genes to be recombined must be within a vector while intra-molecular recombination requires both genes to be within the same vector. The most flexible scheme, presented in Figure 2.1D, allows for recombination anywhere in the genes to be shuffled and is most applicable to antibody engineering because none of the genes must be within the acceptor vector. Thus, pools of antibodies can be shuffled with no bias towards any individual acceptor antibody. To our knowledge, the crossover scheme depicted in Figure 2.1D has not been used to generate antibody libraries in *E. coli.* This fact can be explained by the proficiency of *Saccharomyces cerevisiae* in homologous recombination in comparison to the inherent problems in the endogenous *E. coli* homologous recombination mechanism [49]. For example, the *E. coli* endogenous homologous recombination mechanism is initiated by the cooperation between RecA and the enzyme RecBCD which impedes the use of linear DNA because RecBCD is a vigorous exonuclease [49]. In the cases where *E.coli* has been used to create hybrid genes (Figures 2.1B and 2.1C) mutant strains must be used which are not advantageous because they can exhibit genetic instabilities during library transformation and propagation. Although it is possible to exploit the yeast recombination method to make chimeric genes and then recover the plasmids containing the hybrid gene products for transfer to *E. coli*, this method is hampered by both the inefficient recovery of plasmids from yeast minipreps and the added difficulty of having to switch hosts. Not until the advent of yeast surface display did it become practical to

exploit yeast *in vivo* homologous recombination methods for making chimeric antibody libraries.

This paper is the first report of *in vivo* homologous recombination for the creation of large, yeast surface displayed antibody libraries. Yeast surface display has already proven a powerful tool for affinity maturation by mutagenic PCR of antibodies [16, 31] and T-cell receptors [32] and recently has been shown to be a valuable tool for the isolation of antibodies with novel specificities [50]. Here, we demonstrate that homologous recombination shuffling in conjunction with yeast surface display greatly simplifies the creation of large, chimeric antibody libraries. First, we show that homologous recombination by electroporation reliably generates yeast surface displayed libraries of 10^7 clones and eliminates the need for a ligation step and an *E. coli* cloning step. Next, we use two scFvs that share 89.8% homology between them as a test case to demonstrate that yeast can shuffle genes *in vivo* by homologous recombination. The technique presented in this paper requires fewer PCR cycles than *in vitro* DNA shuffling, does not require a ligation step, and, unlike site specific recombination methods, it allows for recombination anywhere homology exists between the genes to be recombined. Moreover, the diversity generated is not limited to single crossovers, hybrid antibodies created by double and triple crossovers were easily obtained. This technique should prove to be an important addition to the aforementioned methods for the isolation of affinity improved antibodies.

2.2 Materials and Methods

2.2.1 DNA Preparation for Homologous Recombination

The two scFvs used in the shuffling experiments were isolated from a previously constructed yeast surface displayed nonimmune library [50]. To test homologous recombination frequencies of a mutagenic PCR product, a third scFv from this library was amplified in a PCR reaction using a nucleotide analogue mutagenesis procedure that has been previously described [40]. All PCR products were inserted into the vector PCTCON [50] which had been restriction digested from NheI to BamHI (New England Biolabs) and gel purified using a gel purification kit (Qiagen) according to the manufacturer's instructions. As described in Raymond, Pownder and Sexson, to obtain the best transformation efficiency, homologous recombination primers were designed so that the inserts would have approximately a 50 basepair overlap on each end with the cut acceptor vector [44]. The primer used to make inserts with 5' homology to the cut vector was 5'-CGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG-3' and the primer used to make inserts with 3' homology to the cut vector was 5'-CAGATCTCGAGCTATTACAAGTCTTCTTCAGAAATAAGCTTTTGTTC-3'. To make either scFv1 or scFv2 which lacked 5' homology to the cut vector the primer 5'-GCTAGCCAGGTACAGCTGCAGC-3' was used and to make either scFv1 or scFv2 that lacked 3' homology to the cut vector the primer 5'-AATTCCGGATAGGACGGTGAGCTTGG-3' was used. All oligonucleotides were obtained from MWG-Biotech. PCR reactions were carried out on a Perkin Elmer DNA Thermal Cycler 480. A typical PCR reaction was carried out in a 100 μL volume using

10-100 ng of template, 1 μM primers, 0.2 mM of each dNTP, 6.25 U *Taq* (Invitrogen), and 2 mM $MgCl_2$. The cycling conditions used were 1 cycle of 94°C for 1 minute followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, followed by 1 cycle of 72°C for 10 minutes. PCR products were gel purified using a Qiagen kit. Insert fragments were concentrated with Pellet Paint (Novagen) to a concentration of 5 $\mu g/\mu l$ according to the manufacturer's instructions and cut backbone was likewise concentrated to a concentration of 1 $\mu g/\mu l$.

2.2.2 Preparation of Electrocompotent Yeast for Homologous Recombination

The method of yeast preparation closely follows the one described by Meilhoc, Masson, and Teissié [51]. First, 50 ml of YPD was inoculated with the *Saccharomyces cerevisiae* strain EBY100 [16] to OD 0.1 from an overnight culture of EBY100 in YPD. Next, the cells were grown with shaking at 30°C to an OD of 1.3 to 1.5 (about 6 hours of growth). The cells were harvested by centrifugation and resuspended in 50 mL of freshly prepared 10 mM tris, pH 8.0, 25 mM dithiothreitol (DTT) in YPD and shaken for 20 minutes at 30°C. The cells were washed once with 25 ml E buffer (10 mM tris, pH 7.5, 270 mM sucrose, 1 mM $MgCl_2$) and again with 5 ml of E buffer. Finally, cells were suspended in buffer E to give $2 * 10^8$ cells per 50 μl aliquot.

2.2.3 Homologous Recombination Protocol

The ratio of total insert fragment to cut acceptor vector was maintained at 10:1 for all transformations. For shuffling experiments where both scFvs were transformed together, half of the insert fragment pool consisted of one scFv and half of the insert fragment pool consisted of the other. One μg (1 μl) of cut acceptor vector and 10 μg (2 μl) of insert were added to a 50 μl aliquot of electrocompotent yeast and incubated on ice

for 5 minutes. Electroporation was carried out using a Biorad Gene Pulser with a 0.2 cm cuvette (voltage = 0.54kV, capacitance = 25 μF) giving a time constant of about 18 ms. After pulsing, the cell aliquots were transferred to 1 ml of YPD media and incubated for 1 hr at 30°C . Cells were then harvested at 3500 rpm for 4 minutes and resuspended in SDCAA selective media (-Ura, -Trp). A small aliquot of cells was removed and plated on SDCAA plates to determine transformation efficiency.

2.2.4 DNA Isolation and Sequencing

Colonies from the SDCAA plates were grown in 5 mL of SDCAA overnight and the DNA was isolated using a Zymoprep kit (Zymo Research) according to the manufacturer's protocol. Two μl of Zymoprep DNA were used in an XL1-Blue (Stratagene) *E. coli* transformation according to the manufacturer's instructions. Cells were plated on selective LB plates supplemented with 100 mg/L ampicillin. Colonies from these plates were grown overnight at 37°C in LB media plus 100 mg/L ampicillin and DNA was isolated using a Qiagen miniprep kit according to the manufacturer's instructions. DNA was sequenced on a Applied Biosystems model 3730 DNA sequencer using version 3.0 Big Dye chemistry.

2.3 Results

2.3.1 Creation of Large Yeast Surface Displayed Libraries by Homologous Recombination

As has previously been reported, the yeast *S. cerevisiae* is highly efficient at reconstructing plasmids from a linearized plasmid and a PCR product that contains sufficient homology on the 3' and 5' ends [43, 44]. Figure 2.2 outlines the homologous

recombination strategy. The first step involves the linearization of a yeast surface display plasmid, which contains an irrelevant gene flanked by a 5' NheI site and a 3' BamHI site, in a restriction digest. The second step involves the creation of a pool of PCR fragments that share 5' and 3' homology to the cut display vector. Because Raymond, Pownder, and Sexson report that overlaps of $\geq$ 50 basepairs on each end of the PCR product yield the highest number of recombinants [44], we made PCR fragments of mutagenic antibody library DNA that have approximately 50 basepairs of homology to the display vector on their 5' and 3' ends. Cotransformation of these fragments with linearized plasmid allowed for the creation of large yeast displayed libraries. Table 2.1 shows the results of two different transformations of mutagenic antibody library DNA using homologous recombination. Both experiments yielded a transformation efficiency of order of magnitude 10^5 per microgram of insert DNA. Homologous recombination by electroporation simplifies library generation by completely eliminating the need for ligation of PCR products into a cut vector and the subsequent cloning into *E. coli*. Library sizes of 10^7 are routinely achieved without great effort.

2.3.2 *In Vivo* Shuffling of Antibody DNA by Homologous Recombination

It is our desire to extend the technique of homologous recombination in yeast to allow for the easy construction of chimeric scFv antibody libraries. To test if chimeras can be created by homologous recombination, a series of four different transformation experiments (Figure 2.3) were performed using two different antibodies, termed scFv1 and scFv2, which shared 89.8% homology between them. These antibodies, which were isolated from a nonimmune *S. cerevisiae* displayed library [50], were chosen not only because they shared significant patches of homology but also because they had numerous

differences spread throughout the entire length of their sequences (Figure 2.4). These differences are critical for the determination of crossover points. In the experiments depicted in Figures 2.3A and 2.3B, the two genes to be recombined had homology with the linearized display vector at one end only. In principle, neither fragment alone could recreate a whole plasmid because each fragment was missing critical homology on one end. Therefore, only recombinants would be isolated after transformation. To determine if forcing recombination is necessary for the creation of hybrid genes, a third experiment was performed (Figure 2.3C) using scFv1 and scFv2 where both genes contained 5' and 3' homology to the display vector. In a fourth experiment (Figure 2.3D), scFv1 was transformed alone to verify that both 5' and 3' homology are necessary for plasmid reconstruction.

Figure 2.4 shows the results of the *in vivo* homologous recombination shuffling experiments. For all of the results depicted in Figure 2.4, the crossover point in the chimeric antibody sequence was determined to be the last nucleotide of homology 5' to a difference between the chimeric antibody sequence and whichever scFv sequence to which the chimeric antibody had homology up till that point. Because scFv1 and scFv2 share sixty basepairs of homology at the beginning of their sequences and forty four basepairs of homology at the end of their sequences, it is possible for crossovers in these regions to generate genes that have 100% sequence identity to one of the two original scFvs. It was our initial hypothesis that for the insertion into the display vector to be successful, the insert fragment needs both 5' and 3' homology to the cut plasmid. However, the results depicted in Figure 2.4D indicate that this hypothesis is false because ten of the twelve clones sequenced were 100% homologous to scFv1 although scFv1

lacked the 5' homology necessary for *in vivo* homologous recombination to occur. Apparently, the single stranded end of the NheI cut plasmid can recombine with the blunt, double stranded DNA of the scFv to be inserted. It is likely that the BamHI cut end of the vector can also recombine with blunt end DNA in a similar manner. This integration phenomena might be a form of illegitimate integration, defined as recombination involving little or no sequence homology, that has been previously reported to occur in *S. cerivisiae* [52]. Thus, it is impossible to tell if a gene that has 100% homology to either scFv1 or scFv2 is a hybrid or was generated through illegitimate integration. In this paper only genes that clearly contain portions of scFv1 and scFv2 will be considered to be chimeric.

Figure 2.4A (corresponding to the experiment depicted in Figure 2.3A) shows that of the forty eight clones sequenced, twelve were chimeric and Figure 2.4B (corresponding to the experiment depicted in Figure 2.3B) shows that of the forty six clones sequenced, seventeen were chimeric. It is not surprising that all of the hybrids in Figure 2.4A shared homology 5' to the crossover point with scFv2 and homology 3' to the crossover point with scFv1 because in this experiment scFv2 had 5' homology to the cut plasmid and scFv1 had 3' homology to the cut plasmid. As expected, the reverse is generally true for the experiment depicted in Figure 2.3B. We see that in all but two cases (clones 23 and 24) the chimeric clones shared homology 5' to the crossover point with scFv 1 and homology 3' to the crossover point with scFv 2 (Fig. 2.4B). Clones 23 and 24 may have been generated by a crossover event in the first sixty basepairs or may be the result of illegitimate integration. Figures 2.4A and 2.4B indicate that the crossover points are evenly distributed in the hybrids created. Only clones 6 and 18, generated by a

crossover point immediately 5' to CDR3 of the heavy chain, were found at a slightly higher frequency than other chimeras (three times and five times, respectively, in comparison to approximately one occurrence per chimera for the other hybrids).

In Figure 2.4C (corresponding to the experiment depicted in Figure 2.3C), only five of forty six clones sequenced were chimeric. Thus, it appears that forcing the recombination as in the experiments depicted in Figures 2.3A and 2.3B yields a higher frequency of chimeras than when both genes can recombine with the cut vector at both their 5' and 3' ends. The presence of light chain only inserts (clones 33, 34 and 36) is not surprising when one considers that every scFv contains a fifteen amino acid linker consisting of three repeats of four glycines and a serine ($(gly4ser)_3$) that joins the variable heavy and variable light domains. This same linker is present immediately 5' to the gene in the display plasmid and these linkers share 80% sequence homology. It is surprising that these clones were not found in the other two libraries, although there is every reason to believe that further sequencing would reveal the presence of light chain only insertions in both cases. It is important to note that clone 31 resulted from a crossover in a region of two basepairs of homology, indicating that on rare occasions crossovers can occur in regions of very limited homology. This result is in agreement with that found by Mezard, Pompon, and Nicolas [45] and demonstrates that the power of this technique is not limited by the need for long stretches of homology between the genes to be recombined.

Double (clone 24 and 32) and triple (clone 11 and 25) crossovers greatly increase the diversity potential of libraries generated by *in vivo* homologous recombination. To estimate this diversity, we applied the Poisson distribution to estimate the probability p of

obtaining a clone with $x = 0,1,2\ldots$ crossovers in a given sequence by using the following equation:

$$p(x;\lambda) = \frac{e^{-\lambda}\lambda^{x}}{x!} \qquad \textbf{2.1}$$

where λ is defined as the average number of crossovers per sequence in a given experiment. The value of λ was 0.29 in the experiment depicted in Figure 2.3A, 0.43 in the experiment depicted in Figure 2.3B, and 0.13 in the experiment depicted in Figure 2.3C. For each of these experiments the expected and actual number of clones obtained are listed in Table 2.2. As one can clearly see, the number of clones experimentally obtained for $x = 0$ or $x = 1$ is well approximated by the Poisson distribution. The limited sampling size of between forty five and fifty clones per experiment explains the lack of agreement for $x = 2$ and $x = 3$. Table 2.3 gives the expected number of clones containing a given number of crossovers for a $1 * 10^7$ library. Although this table gives a picture of the distribution of crossovers, it does not take into account the number of unique sequences which is the true measure of library diversity. The following equation can be used to determine the number of unique sequences, u, obtained with a given number of crossovers x when y genes are crossed:

$$u = y(y-1)^{x}\left(\frac{z!}{x!(z-x)!}\right) \qquad \textbf{2.2}$$

where $\left(\frac{z!}{x!(z-x)!}\right)$ indicates the number of combinations with z crossover points. As shown in Table 2.3, for a cross of two genes with an arbitrarily chosen number of twenty

distinct crossover points this equation yields 40 unique one crossover clones, 380 unique two crossover clones, and 2280 unique three crossover clones. The data in Table 2.3 reveals the trend that the number of unique clones with multiple crossovers will become greater than the number of clones predicted to have multiple crossovers by a Poisson distribution as the number of genes crossed is increased. For crosses of small numbers of antibodies, the chimeras in the library created would be dominated by many copies of each unique single crossover clone but the added diversity created by double and triple crossovers would be nonzero. For a cross of one hundred genes, the potential theoretical diversity would be dominated by unique multiple crossover clones and would greatly exceed the diversity of multiple crossover clones predicted to be in the library by the Poisson distribution, however, the unique single crossover clones would still be oversampled in a $1 * 10^7$ library. In addition, the number of double and triple crossover clones as predicted by the Poisson distribution, 77910, is 39% of the maximum number of unique single crossover clones, 198000, indicating that double and triple crossovers are a nontrivial component of the total diversity. Of course, clones with four or more crossovers may exist, but because none were isolated in the limiting sampling of this experiment they were not included in the analysis.

The possibility that forcing recombination reduced transformation efficiency below a level that was useful was considered. However, for all of the crosses performed here the transformation efficiency was of order 10^4 to 10^5 per microgram of insert. Thus, forcing recombination does not appreciably affect transformation efficiency.

2.4 Discussion

In this paper, we present a simple method for reliably producing large, yeast surface displayed chimeric antibody libraries. This method takes advantage of the homologous recombination pathway in yeast to reconstruct full plasmids from restriction digested plasmids and PCR products that have 5' and 3' homology to that cut plasmid. Transformation of multiple PCR products that share homology can cause recombination events to yield chimeric gene products. Forcing recombination by making a PCR product of one gene with 5' homology to a cut vector and making a PCR product of a second gene with 3' homology to a cut vector yields greater numbers of chimeric gene products than when both genes to be recombined share both 5' and 3' homology to the vector into which they are to be inserted. The transformation efficiency for forced recombination was not appreciably lower than that of a single PCR product with both 5' and 3' homology to the cut display plasmid.

Although forced recombination yields the greatest number of hybrid genes, this method may prove impractical because one must know the sequence of the 5' or 3' ends of the genes that are to be shuffled. In the case presented in this paper, the two genes to be recombined had known sequences that facilitated primer design. However, one may wish to shuffle one scFv gene against an entire library of scFvs or shuffle a batch of scFv genes that all bind a given antigen. Generic primer sets for all antibody heavy and light chains are known and it would be possible to force recombination by creating PCR products that lack 5' or 3' homology to the vector into which the genes are to be inserted. This method is rather tedious and in our experience has proven unnecessary. As our experiments demonstrate, not forcing recombination allows for the creation of hybrid

clones, albeit at a lower rate than forced recombination. The number of chimeric clones obtained is only two to three fold less than in forced recombination and is therefore not low enough to cause concern that forcing recombination is necessary for this technique to be practical.

It is important to stress that the ability for as little as two nucleotides homology to yield successful recombinants in conjunction with the presence of double and triple crossovers demonstrates that this technique has a vast potential for producing highly diverse libraries. Although it is true that most chimeras contain only one crossover, there will still be many unique clones in the library to ensure the necessary diversity for directed evolution experiments. It is likely that the unique diversity of libraries made by yeast *in vivo* homologous recombination will be sufficient for affinity improvements even if the actual diversity of the libraries generated is orders of magnitude lower than the total number of transformants because the shuffling of antibodies is a rearrangement of diversity that has been maintained specifically because it is functional. The advantage of this technique is its simplicity and it is likely that many variations other than those presented here will emerge for the production functional diversity in protein libraries.

Table 2.1: Transformation efficiencies of two different homologous recombination experiments.

Trial	Insert to Vector Ratio	Micrograms of Insert Transformed	# of Transformed Clones	Transformants per Microgram of Insert
1	10:1	31	$1.8 * 10^7$	$5.8 * 10^5$
2	10:1	60	$2.3 * 10^7$	$3.8 * 10^5$

Table 2.2: Expected and experimentally determined number of clones with a given number of crossovers as predicted by a Poisson distribution.

	scFv1 with 3' Homology + scFv2 with 5' Homology		scFv1 with 5' Homology + scFv2 with 3' Homology		scFv1 with 5' and 3' Homology + scFv2 with 5' and 3' Homology	
Number of Crossovers per Sequence (x)	Actual Number of Sequences with x Crossovers	Poisson Calculated Number of Sequences with x Crossovers	Actual Number of Sequences with x Crossovers	Poisson Calculated Number of Sequences with x Crossovers	Actual Number of Sequences with x Crossovers	Poisson Calculated Number of Sequences with x Crossovers
0	36	36	29	30	41	40
1	11	10	15	13	4	5
2	0	2	1	3	1	0
3	1	0	1	0	0	0

Table 2.3: Expected number of total clones and unique clones with a given number of crossovers as predicted by a Poisson distribution for a $1 * 10^7$ library.

Number of Crossovers per Sequence (x)	Number of Sequences with x Crossovers in a $1 * 10^7$ Library with $\lambda = 0.13$	Number of Unique Sequences Assuming 20 Distinct Crossover Points			
		Recombine 2 Genes	Recombine 3 Genes	Recombine 4 Genes	Recombine 100 Genes
0	8777137	2	3	4	100
1	1144844	40	120	240	198000
2	74664	380	2280	6840	186219000
3	3246	2280	27360	123120	$1.1 * 10^{11}$
Total	$\approx 1 * 10^7$	2702	29763	130204	$1.1 * 10^{11}$

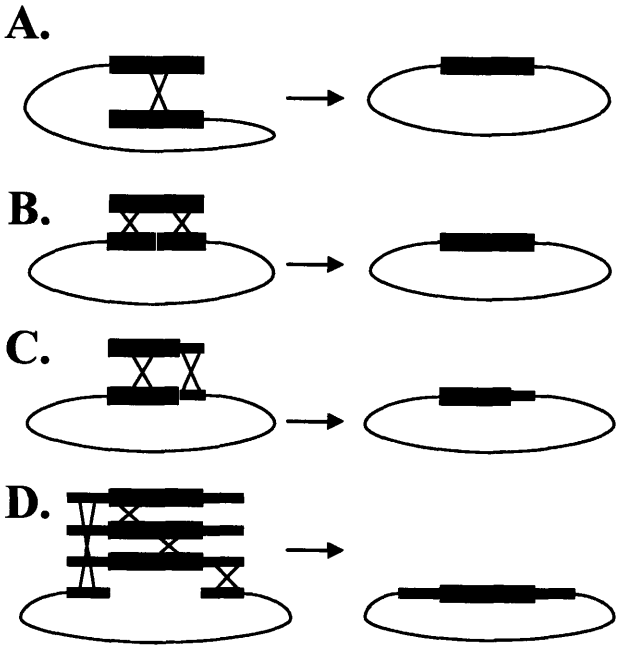


Figure 2.1: Some common *in vivo* homologous recombination schemes. (A) Intra-molecular recombination where both genes to be recombined are located within the same plasmid. (B) Inter-molecular recombination between a gene and a similar gene that is located within a plasmid and has been cut at an internal restriction site. The acceptor gene within the plasmid determines he 5' and 3' ends of the gene hybrid. (C) Inter-molecular recombination between a donor gene that contains a portion of homology on its 3' end to the vector to which it is being inserted. The vector is cut at the 3' end of the acceptor gene that is located within the plasmid. The acceptor gene determines the 5' end of the hybrid gene generated. (D) Multiple gene hybrids are generated by genes that contain homology at their 5' and 3' ends to the vector to which they are to be inserted. Homology within the genes allows for multiple recombination events and the order of the genes within the final hybrid is not fixed by the presence of a gene within the acceptor plasmid. This figure is adapted from Wang [48].

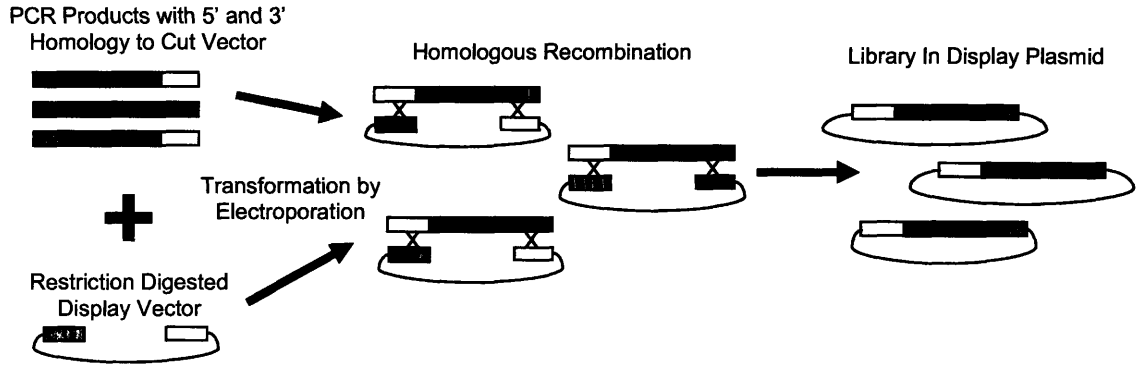


Figure 2.2: General outline of library construction using homologous recombination in yeast. First, PCR products are created with 5' and 3' homology to the vector into which they are to be inserted. Next, the PCR products are transformed together with a restriction digested vector by electroporation, and yeast utilizes the homology between the cut vector and the PCR products to reconstruct whole plasmids.

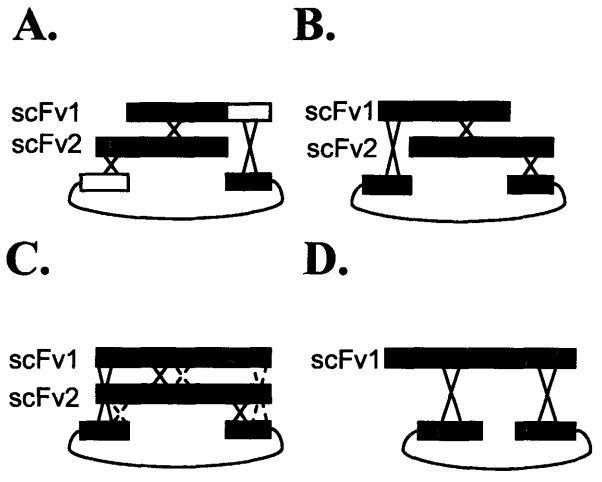


Figure 2.3: *In vivo* homologous recombination experiments. (A) Homologous recombination when scFv 1 has 3' homology to the display vector and scFv 2 has 5' homology to the display vector. (B) Homologous recombination when scFv 1 has 5' homology to the display vector and scFv 2 has 3' homology to the display vector. (C) Homologous recombination when both scFv 1 and scFv 2 have 5' and 3' homology to the display vector. Solid lines indicate a crossover producing chimeric clones that have 5' homology to scFv 1 and 3' homology to scFv 2. Dashed lines indicate a crossover to produce chimeric clones with the opposite arrangement. A mixed path of one solid line crossover and one dashed line crossover without crossover between the two scFvs would produce a plasmid without a chimeric gene. (D) Homologous recombination when scFv 1 has 3' homology to the display vector and scFv 2 is not present. This recombination occurs between the $(gly4ser)_3$ linker that joins the scFv heavy and light chains and the $(gly4ser)_3$ linker located immediately 5' to the ultimate location of the gene in the display plasmid.

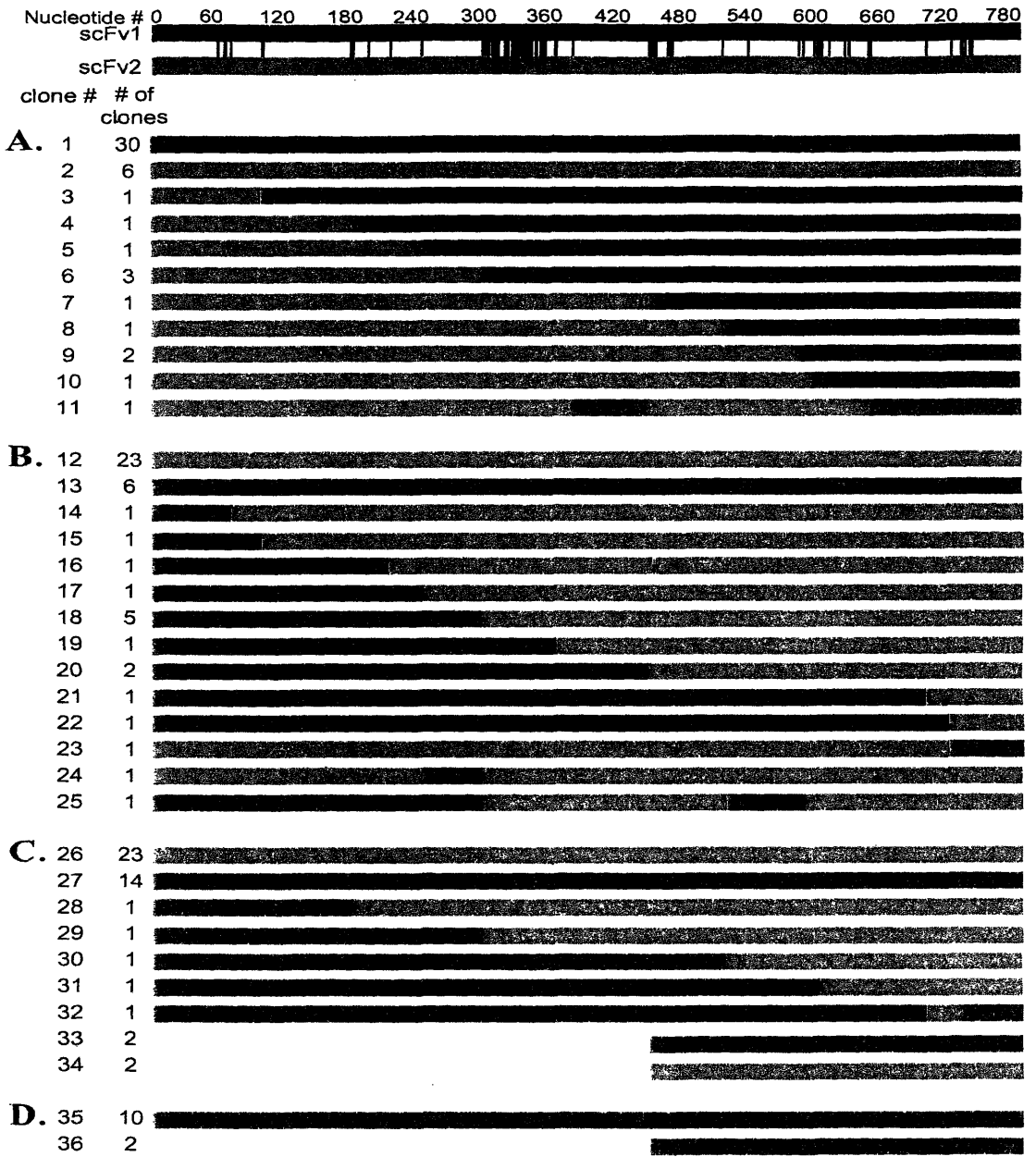


Figure 2.4: Results of homologous recombination shuffling experiments. scFv 1 is shown in blue, scFv 2 is shown in red, and the differences between them are indicated by black bars connecting the two. (A) Chimeric clones produced by homologous recombination of scFv 1 with 3' homology to the cut vector and scFv 2 with 5' homology to the cut vector. (B) Chimeric clones produced by homologous recombination of scFv 1 with 5' homology to the cut vector and scFv 2 with 3' homology to the cut vector. (C) Clones produced when both scFv 1 and scFv 2 have 5' and 3' homology to the cut vector. (D) Clones produced when only scFv 1 with 3' homology to the cut vector is transformed.

Chapter 3: Integrated Mimicry of B Cell Antibody Mutagenesis by PCR and Homologous Recombination in Yeast

3.1 Introduction

B cells utilize a variety of mechanisms to adaptively generate antibody sequence diversity. Preimmune V(D)J recombination first creates CDR3 loops of varying length and composition[53]. Then, in response to antigen challenge, the antibody repertoire is further shaped by somatic hypermutation (SHM)[54], combinatorial replacement of entire light chains (receptor editing[6-8]), and exchange of portions of the V_H gene (receptor revision[9, 10]). Light chain exchange in the periphery has been observed in humans[7] and mice[8], and has been proposed to play a role in fine tuning antigen binding affinity[55]. Receptor revision creates hybrid V_H genes that maintain the same CDR3 while splicing in new coding sequence for FR1, CDR1, FR2, CDR2, and most or all of FR3[56, 57]. Receptor revision and receptor editing cause larger movements in sequence space than SHM, and are hypothesized to enable the affinity maturation process to escape local energetic minima[58]. Amino acid insertions and deletions in CDRs occur frequently *in vivo*[3-5], and such changes are well tolerated by the variable region's framework architecture[59]. The broad spectrum of mutational strategies employed by B cells ensures a thorough search of sequence space surrounding the critical V_H CDR3 residues that dominate antigen recognition in the preimmune repertoire[60, 61].

In contrast, *in vitro* antibody affinity maturation strategies to date have employed mutagenesis with a much narrower scope. Diverse antibody libraries for affinity

maturation have been generated by random mutagenesis throughout the entire variable regions[30, 31, 62], gene shuffling[31], targeted mutagenesis of CDR residues[63, 64], light chain shuffling[42, 65], and CDR shuffling[42, 65, 66]. Most *in vitro* antibody affinity maturation strategies employ error-prone PCR for mutagenesis, which can sample a varying frequency of nucleotide point mutations[30]. Random mutagenesis in conjunction with gene shuffling[31] and targeted mutagenesis of CDR residues[63] have shown to yield dramatic affinity improvements of 3 orders of magnitude or greater. Methods that involve chain or CDR shuffling have proven less successful, with affinity improvements up to 300 fold when directed against a hapten antigen[42] and up to 6 fold when directed against a protein antigen[65, 66]. The mutagenesis methods just enumerated have been used previously in isolation, or in pairs. However, for a given antibody/antigen pair it is not possible to identify *a priori* the particular categories of genetic diversity from which the greatest affinity improvements will be isolated.

In this study, we have developed an integrated approach combining error prone PCR and *in vivo* homologous recombination in yeast that mimics the full spectrum of mutagenesis strategies employed by B cells: random point mutagenesis, amino acid insertions and deletions, receptor revision, and receptor editing. This approach has been applied to increase the affinity of an anti-peptide antibody by at least five orders of magnitude, from μM to pM affinity, in three library construction and screening steps. Facile access to sequence diversity beyond point nucleotide mutations mimics the approach used by the humoral immune system to ensure a robust adaptive response against effectively any macromolecular antigen presented on a pathogenic surface. In addition, this multiplexing affinity maturation strategy decreases the likelihood of

convergence upon a single high affinity solution, allowing for the generation of multiple high affinity binders, with potentially different secretory properties and therapeutic potential.

3.2 Materials and Methods

3.2.1 Materials

The non-immune library used to isolate anti-PSGL-1 binders has been previously described[50]. The divalent, biotinylated b.19.ek.Fc (a biotinylated bivalent fusion of the N-terminal 19 amino acids of PSGL-1 to a human Fc) protein and the human Fc were generous gifts of Dr. Raymond T. Camphausen, formerly of Wyeth (Cambridge, MA)[24]. The monovalent from of this antigen lacking the Fc portion, b.19.ek, was a gift of Gray Shaw (Wyeth, Cambridge, MA). The yeast strain used for all experiments, EBY100, has been previously described[16]. The yeast surface display vector PCTCON, a derivative of the vector PCT302, has also been previously described[50].

All restriction enzymes and ligases were purchased from New England Biolabs (Beverly, MA). Mouse monoclonal antibody, 9e10, directed against the c-myc epitope tag, was purchased from Covance (Richmond, CA). The fluorophores goat anti-mouse FITC, goat anti-biotin FITC, and goat anti-mouse PE were purchased from Sigma (St. Louis, MO) and streptavidin-PE was purchased from PharMingen (San Diego, CA).

3.2.2 Mutagenic PCR

Mutagenic PCR of the anti-PSGL-1 scFv was performed using the nucleotide analogues 8-oxo-2'-deoxyguanosine-5'-triphosphate (8-oxodGTP) and 2-deoxy-P-nucleoside-5'triphosphate (dPTP), which were both purchased from TriLink

Biotechnologies (San Diego, CA)[40, 67]. Primers were ordered from MWG Biotech (High Point, NC). The forward primer was 5'-CGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG-3' and reverse primer was 5'-CAGATCTCGAGCTATTACAAGTCTTCTTCAGAAATAAGCTTTTGTTC-3'. These primers have approximately a 50 basepair overlap with PCTCON that has been digested from NheI to BamHI. PCR reactions were carried out on a Perkin Elmer DNA Thermal Cycler 480. All mutagenic PCR reactions were carried out in a 100 μL volume using 10-100 ng of template, 1 μM primers, 0.2 mM of each dNTP, 6.25 U *Taq* (Invitrogen, Carlsbad, CA), and 2 mM $MgCl_2$. To vary the mutation rate, both the concentration of the analogues and the number of PCR cycles was varied. Six separate PCRs were performed: 5 cycles with 200 μM analogues, 10 cycles with 200 μM analogues, 10 cycles with 20 μM analogues, 10 cycles with 2 μM analogues, 20 cycles with 20 μM analogues, and 20 cycles with 2 μM analogues. The cycling conditions used were 1 cycle of 94°C for 1 minute followed by either 5, 10 or 20 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, followed by 1 cycle of 72°C for 10 minutes. PCR products were gel purified using a Qiagen (Valencia, CA) kit. After purification, a 1:50 dilution of the 20 cycle purified PCR product and a 1:10 dilution of the 10 cycle PCR product were prepared and 5 μLs were used in a 100 μL PCR reaction with the following cycling conditions: 1 cycle of 94°C for 1 minute followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, followed by 1 cycle of 72°C for 10 minutes. For the 5 cycle PCR purified product, the PCR was performed in the same way except the purified template was not diluted. All amplifications were performed using the same

concentrations of reagents as in the mutagenic PCR except the nucleotide analogies were omitted. The PCR amplified products were gel purified using a Qiagen kit.

3.2.3 Receptor Revision and Receptor Editing Libraries of Anti-PSGL-1 scFvs.

The libraries where the V_H CDRs were preferentially replaced were created from truncated PCR products based upon r2s4-2. Library RR1 was created by cotransforming a r2s4-2 PCR product that lacked the first framework region and CDR of the V_H with a pool of PCR products generated from non-immune library DNA. Library RR2 was created in a similar manner except the r2s4-2 PCR product lacked the first two CDRs and first two framework regions. The fact that the PCTCON vector contains a BamHI cut site 5' to the c-myc tag and a XhoI site 3' to the c-myc tag was used to facilitate library construction. To force recombination, it was necessary to restrict 5' homology to the cut vector to the pool of non-iummune library PCR products and to restrict 3' homology to the cut vector to the r2s4-2 PCR products. The forward primer HA-for, 5'-GTTCCAGACTACGCTCTGCAG-3', which primes in the HA region of PCTCON and is well 5' of the NheI cut site that begins every scFv, was used to generate non-immune library PCR products for the RR1 and RR2 libraries. The reverse primer used to generate non-immune library based PCR products, c-myc-library-rev, 5'-CTTCTTCAGAAATAAGCTTTTGTTC-3', primes in the c-myc region and creates fragments that lack 3' homology to an NheI to XhoI digested PCTCON backbone. For library RR1, r2s4-2 based PCR products were created using the forward primer FR2-for, 5'-GGATCAGGCAGTCCCCATCG-3', which primes in framework 2 and for library RR2, r2s4-2 based PCR products were created using the forward primer FR3-for, 5'CGAATAACCATCAACGCAGAC-3', which primes in framework 3. The reverse

primer in both cases was past-XhoI-rev, 5'-GGGAACAAAGTCGATTTTGTTAC-3' which primers 3' to the XhoI site. The cutting of the backbone PCTCON from NheI to XhoI ensured that the non-immune library fragment would insert on its 5' end and the r2s4-2 fragment would insert on its 3' end because neither fragment alone would have the necessary homology to form a complete plasmid.

The receptor editing library was created in a slightly different manner. The PCTCON backbone was digested from NheI to BamHI. A V_H only fragment of r2s4-2 was generated using the HA-for primer and a reverse primer V_L-rev, 5'-GGAGACTGTGTCAACACAATTTC-3', that primes 3' to the $(gly_4ser)_3$ linker. The non-immune library DNA was PCRed using the HA-for primer and a reverse primer c-myc-rev, 5'-CAGATCTCGAGCTATTACAAGTCTTCTTCAG-3'. The non-immune library PCR products for the creation of a receptor editing library were digested from NheI to BamHI to eliminate the 5' homology to the cut PCTCON. There is a conserved BamHI site in most scFvs 5' to the $(gly_4ser)_3$ linker thus cutting with BamHI generated a pool of light chain fragments.

All PCR reactions were carried out in a 100 μL volume using 10-100 ng of template, 1 μM primers, and 0.2 mM of each dNTP. For the amplification of fragments based upon r2s4-2, 2.5 U of *PfuTurbo* (Stratagene, La Jolla, CA) were used to decrease the likelihood of introducing mutations. For amplification of non-immune library DNA, 6.25 U of *Taq* and 2 mM $MgCl_2$ were used. The cycling conditions were 1 cycle of 94°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.5 minutes, followed by 1 cycle of 72°C for 10 minutes. All PCR fragments were purified using a Qiagen PCR purification kit

3.2.4 Yeast Transformation

Insert fragments were concentrated with Pellet Paint (Novagen, Madison, WI) to a concentration of 5 $\mu g/\mu l$ according to the manufacturer's instructions and cut PCTCON backbone was likewise concentrated to a concentration of 1 $\mu g/\mu l$. The insert to backbone ratio was 10:1 on a mass basis. For the receptor revision and receptor editing libraries the insert was an equal mix of non-immune library PCR product and r2s4-2 PCR product. The method for the preparation of electrocompotent yeast and their transformation has been described previously[68]. Transformation efficiencies were on the order of 10^5 transformants per microgram of insert DNA.

3.2.5 Yeast Growth and Induction

Detailed protocols for yeast growth and induction can be found in Boder and Wittrup, 2000[69].

3.2.6 Flow Cytometry for Sorting and K_d Determination on the Yeast Surface

All sorting was performed on a MoFlo cell sorter (Cytomation, Fort Collins, CO). For the lead isolation round of anti-PSGL-1 scFvs, a subset of 2.3 * 10^8 clones from a library with overall diversity of 1 * 10^9 clones[50] was sorted for potential binders. Three total sorts were performed at a concentration of 800 nM b.19.ek.Fc and, to prevent isolating fluorophore binders, goat anti-biotin FITC and streptavidin-PE were alternately used to detect b.19.ek.Fc binding clones. In addition, 10 fold excess human Fc was added as a competitor in the second and third sorts to prevent isolation of Fc binders. The DNA of all sixteen clones isolated from round 0 were pooled and mutagenized together. This new pool of mutant DNA was transformed into yeast and plasmids were recreated using *in vivo* homologous recombination[44] to create a round 1 library of $4.8*10^6$ clones. The library was sorted six times at a concentration of 100 nM b.19.ek.Fc for sorts

1-4, 50 nM b.19.ek.Fc for sort 5, and 25 nM b.19.ek.Fc for sort 6. For round 2, a library of 1.8 * 10^7 was created by *in vivo* homologous recombination and sorted four times using 50 nM b.19.ek.Fc for sort 1, 25 nM b.19.ek.Fc for sort 2, 10 nM b.19.ek.Fc for sort 3, and 5 nM b.19.ek.Fc for sort 4. For round 3, the random mutagenic library contained 2.3 *10^7 clones and was 13.5 times larger than the receptor editing library and 77 fold larger than two receptor revision libraries. All four of these libraries were sorted four times separately using a concentration of 5 nM divalent b.10.ek.Fc for the first sort, 5 nM b.19.ek.Fc plus 33 fold excess KPL1 for the second sort, 5 nM monovalent b.19.ek for the third sort, and a concentration of 2.5 nM b.19.ek for the fourth sort.

Between sort analysis and titrations for determining scFv K_ds were performed on a Coulter EPICS XL (Miami, Fl). For analysis, approximately 2 * 10^6 yeast were suspended in 100 μl of PBS + 0.1% BSA (to be referred to as PBS in this paper). Cells were labeled with either b.19.ek.Fc or b.19.ek at the appropriate concentration for ½ hour at 37°C. After a wash with 1 mL of cold PBS, the yeast were incubated ½ hour on ice with 9e10 in a 1:100 dilution in PBS. After a cold PBS wash, the yeast were incubated ½ hour on ice with either goat anti-mouse FITC in a 1:50 dilution plus streptavidin-PE in a 1:100 dilution in PBS or goat anti-mouse PE in a 1:25 dilution and goat anti-biotin FITC in a 1:50 dilution in PBS. For sorting, these labeling conditions were scaled appropriately for the increased number of yeast. The method for the determination of optimum labeling concentrations and sort windows has been shown earlier[69]. The method for performing a titration to determine the K_d of yeast surface displayed scFvs has also been described previously[69].

3.2.7 Representation of the Electrostatic Potential at the Surface of Clone RR2r3s4-1

The model of the antibody RR2r3s4-1 was created using the website Web Antibody Modeling (antibody.bath.uk.ac.uk). The solvation potential at the surface was computed by solving the Poisson-Boltzmann equation (inner dialectric constant = 4, outer dialectric constant = 80) using an in-house modified version of the program DELPHI (Tidor Group, Massachusetts Institute of Technology, Cambridge, MA). It was modeled using CHARMM19. Special thanks to Michael D. Altman for the software used to plot the electrostatic potential at the surface.

3.3 Results

3.3.1 Lead Isolation Round 0

A previously described[50] nonimmune antibody displayed on the surface of yeast was sorted for binders to a biotinylated bivalent fusion of the N-terminal 19 amino acids of PSGL-1 to a human Fc (b.19.ek.Fc)[24]. This construct contains the portion of PSGL-1 that has been determined to be both necessary and sufficient for adhesion to P-selectin[24] and is an important drug target for the development of anti-inflammatory therapeutics[70] (Figure 3.1). After three sorts, a sample of sixteen clones was sequenced and all were found to be unique (data not shown). The pool of clones was verified to be specific for b.19.ek.Fc because the pool did not show decreased binding in the presence of ten fold excess Fc and did not bind fluorophores in the absence of b.19.ek.Fc. The bivariate histogram showing the binding of the best clone from this round can be seen in Figure 3.2A. The K_d values for clones isolated in this round are difficult to determine because of lack of sufficient b.19.ek.Fc to create the necessary concentrations needed to measure the K_d; additionally, at concentrations greater than 1 μM nonspecific ligand stickiness to the

yeast's surface becomes problematic. However, a lower bound K_d estimate of greater than 5 μM (Figure 3.3) was made from an examination of binding data at concentrations below saturation (data not shown). The CDR amino acid sequence of two of the clones from this round, r0s3-6 and r0s3-15, are shown in Table 3.1. The best binding clone from this round was not r0s3-6 or r0s3-15, the two clones that provided genetic material for the subsequent affinity maturation rounds.

3.3.2 Affinity Maturation Round 1 Utilizing Random Mutagensis and Receptor Editing

After six sorts, a single clone, termed r1s6-15, was isolated from a library made of pooled DNA from round 0 that had been mutagenized and transformed together. This clone displayed much better than the best clone from the previous round (Figure 3.2A and 3.2B). Surprisingly, this clone was not entirely derived from any single clone from the lead isolation round but was a hybrid with a V_H that had a 99.5% match to the r0s3-6 V_H and a V_L that had a 100% match to r0s3-15 V_L (Table 3.1 and Figure 3.3) indicating that receptor editing had occurred through *in vivo* homologous recombination within the $(gly_4ser)_3$ linker region shared by all scFvs. The only mutation was glutamic acid to glycine in V_HCDR3.

Again, an accurate K_d of clone r1s6-15 was not determined, however an estimate of a K_d of between 1 and 5 μM (Figure 3.3) was made from an examination of binding data at concentrations below saturation (data not shown). Specificity for the N-terminal 19 amino acids of PSGL-1 was verified by observing the absence of b.19.ek.Fc binding to r1s6-15 displayed on the surface of yeast in the presence of a 10 fold excess of the commercially available anti-PSGL-1 antibody KPL1 which has been shown to bind the desired epitope [71] (Figure 3.4A and 3.4B). In addition, r1s6-15 did not show reduced

binding to b.19.ek.Fc in the presence of a 10 fold excess of an anti-enterokinase binding monoclonal antibody (Figure 3.4A and 3.4C) or a 30 fold excess of a human Fc (Figures 3.4D and 3.4E).

3.3.3 Affinity Maturation Round 2 Utilizing Random Mutagensis

The best clone from round 1 was subject to random mutagenesis throughout the entire gene. Ultimately, one clone was isolated with three mutations, one in the linker region, one in the V_HCDR3, and one in the V_LCDR3 (Table 3.1). For this clone, both the monovalent affinity, 50 ± 30 nM, and divalent affinity, 90 ± 110 pM, were determined (Figure 3.3). This data allows for the affinity improvement due to avidity to be estimated using the theory of Jencks[72]. The essence of this theory is that the K_d for the binding of two tethered binding sites A and B, K_d^{AB}, is not equal to the product of the K_d for the binding of A to its ligand, K_d^A, times the K_d for the binding of B to its ligand, K_d^B:

$$K_d^{AB} \neq K_d^A K_d^B \qquad \textbf{3.1}$$

Equation 3.1 cannot be valid because the units are equal to concentration on the left side of the equation and concentration squared on the right side of the equation. It is possible to define a unitless K_d, K_u, that makes Equation 3.1 valid:

$$K_d^{AB} = K_d^A K_u^B \qquad \textbf{3.2}$$

This unitless K_d is defined as

$$K_u^B = \frac{K_d^B}{[A/B]_{eff}} \qquad \textbf{3.3}$$

where $[A/B]_{eff}$ is the effective concentration of B given that A has already bound. Substituting equation 3.3 into 3.2 we get

$$K_d^{AB} = \frac{K_d^A K_d^B}{[A/B]_{eff}} \quad \textbf{3.4}$$

The protein b.19.ek.Fc has two identical binding sites so

$$K_d^A = K_d^B \quad \textbf{3.5}$$

$$[A/B]_{eff} = [A/A]_{eff} \quad \textbf{3.6}$$

$$K_d^{AB} = K_d^{AA} \quad \textbf{3.7}$$

Therefore, substituting equations 3.5, 3.6, and 3.7 into 3.4 we get

$$K_d^{AA} = \frac{K_d^{A^2}}{[A/A]_{eff}} \quad \textbf{3.8}$$

The K_d for divalent binding, K_d^{AA}, will be enhanced by the increased local concentration of A due to avidity when one A is already bound as long as K_d^A is less than $[A/A]_{eff}$. When $K_d^A = [A/A]_{eff}$, the divalent K_d^{AA} is equal to the monovalent K_d^A and there is no avidity effect. Using the data from round 2 the effective concentration of PSGL-1 that is tethered to already bound PSGL-1 is equal to 28 μM. The display level on the yeast surface affects the local environment and therefore affects the effective concentration. Because the round 0 clones are less well displayed than those in subsequent rounds (Figure 3.2), the effective concentration in round 0 is likely to be less than 28 μM. However, using an effective concentration of 28 μM gives an estimate of the lower bound of the monovalent K_d in round 0. Substituting the divalent K_ds for round 0 and 1 and the effective concentration of 28 μM into the Jencks equation, the monovalent K_d for round 0 and 1 can be estimated to be in the low micromolar range (Figure 3.3).

3.3.4 Affinity Maturation Round 3 Utilizing Random Mutagensis, Receptor Revision, and Receptor Editing

To compare receptor revision, receptor editing, and error-prone PCR random mutagenesis, four separate libraries were created, all based upon r2s4-2. The random mutagenic library was 13.5 times larger than the receptor editing library (Figure 3.5C) and 77 fold larger than two receptor revision libraries (Figure 3.5A and 3.5B). Two different receptor revision libraries were made using *in vivo* homologous recombination, one (RR1) that biased crossovers to the FR2 region leading to a preferential replacement of r2s4-2's V_HFR1 and V_HCDR1 (Figure 3.5A) and one (RR2) that biased crossovers to the FR3 region leading to preferential replacement of r2s4-2's V_HFR1, V_HCDR1, V_HFR2, and V_HCDR2 (Figure 3.5B). The receptor editing library, RE, was made to replace only the light chain with new light chains from the nonimmune library (Figure 3.5C). In all of these libraries, V_HCDR3 was maintained because previous studies have indicated that this domain is in the center of the antigen binding site and is often significant in determining antibody specificity[60, 61]. Our strategy was to alter regions in the binding site periphery such as the V_HCDR1, both the V_HCDR1 and V_HCDR2, and the light chain in order to further optimize the binding site. This approach is analogous to the genetic diversity mapped onto antibody binding sites following somatic hypermutation and affinity maturation *in vivo*[61]. Diversity in these libraries is generated from nonimmune library DNA which codes for sequences already selected as being both compatible with the antibody fold and presumably functionally expressed *in vivo*.

Table 3.2A shows that for a random sampling of ten clones from the RR1 library six had a new CDR1 but maintained the original V_HCDR2 and V_HCDR3, indicating that the crossover occurred in FR2 as designed. Two clones had new heavy chains but were

from the same V_H6-1 germline gene as the V_H in r2s4-2. One clone had a new heavy chain altogether. From this data, it appears that roughly half of the hybrid genes created had a crossover in FR2. Table 3.2B indicates that for a random sampling of ten clones from the RR2 library, four had a crossover in FR3 leading to clones with a new V_HCDR1 and V_HCDR2 but maintained the V_HCDR3. Four clones had new heavy chains and two clones not shown in the table were V_L only. Light chain only clones were generated by a crossover between the $(gly4ser)_3$ linker 5' to the light chain and the $(gly4ser)_3$ linker in the display plasmid 5' to the region coding an scFv. Thus, roughly half of the clones in RR2 had new sequences 5' to the r2s4-2 V_HCDR3.

Table 3.2C shows that four of ten clones sampled from the receptor editing library had new light chains. Three had new κ chains and one new chain was a λ chain. Six of the clones contained only a light chain or a duplicate of the r2s4-2 heavy chain and a new light chain. In a similar manner to light chain only clone formation, clones with duplicate r2s4-2 V_Hs were generated by a crossover of a V_H with the $(gly4ser)_3$ linker of a second V_H which then crosses over with the $(gly4ser)_3$ linker 5' to the V_L. Again, roughly half of the clones had a single V_H and a new V_L.

All of the libraries except the RE library yielded affinity improvements over r2s4-2 (Figure 3.2F-J). The mutagenic library best clone, called r3s4-3, had a K_d for monovalent b.19.ek of 180 ± 100 pM, the RR1 library best clone, called RR1r3s4-2, had a K_d for b.19.ek of 220 ± 200 pM, and the RR2 library best clone, called RR2r3s4-1, had a K_d for b.19.ek of 70 ± 60 pM (Figure 3.3). The total monovalent affinity improvement after three rounds of mutagenesis is estimated to be at least five orders of magnitude.

Sequencing revealed three mutations in the mutagenic library best clone, one in V_HCDR2, one in V_HCDR3, and one in V_LCDR2 (Table 3.1). The RR1 library best clone had three mutations in V_HCDR1 and an insertion of an arginine into V_HCDR2 (Table 3.1). The RR2 library best clone also had three mutations in V_HCDR1, although only two were different than the three found in the V_HCDR1 of the best clone from RR1, the same arginine insertion found in the best clone from RR1, and three additional mutations in V_HCDR2 (Table 3.1). The discovery of an arginine insertion in V_HCDR2 is a result of the receptor revision method and would not be found through error-prone PCR mutagenesis. The ability to easily isolate beneficial insertions and deletions is an important advantage of the receptor revision method.

3.3.5 Framework Mutations in Isolated Clones

It is important to know how many mutations the framework regions of each scFv have because it is common belief in industry that antibodies with framework mutations are more likely to be immunogenic than those that do not contain framework mutations. The sequence of each scFv is compared to the framework sequences in Appendix A. The clone r1s6-15 has five framework mutations, three in the heavy chain and two in the light chain. These same two light chain framework mutations appear in all subsequent clones. Moreover, clones r2s4-2 and r3s4-3 have the same heavy chain framework mutations as in r1s6-15. Clone RR1r3s4-2 has the same mutations as in the r1s6-15 heavy chain framework regions plus one more mutation in V_HFR1. Clone RR2r3s4-1 also has four total mutations in the heavy chain framework regions but only one is the same as in clone RR1r3s4-2.

It was not determined whether or not any of the framework mutations were essential to the affinity of a given clone. If necessary, site directed mutagenesis could be used to change each framework mutation back to the amino acid present in the germ line.

3.3.6 Electrostatic Potential at the Surface of Clone RR2r3s4-1

The electrostatic potential at the surface of clone RR2r3s4-1 is shown in Figure 3.6. This figure is based upon a hypothetical model and is not an actual crystal structure. Therefore, the orientation of the residues may not be exact and the residues may have a different orientation when bound to PSGL-1. However, the model proves useful in demonstrating the charge distribution in the binding site. It is immediately apparent that the binding site of the antibody is very blue in comparison with the rest of the antibody, indicating that this region is of positive electrostatic potential. Since this antibody binds to a peptide that contains negatively charged sulfotyrosines it makes sense that the antibody binding site would be positively charged. A number of arginines, including Arg31, Arg94, Arg162, Arg166, Arg167, and Arg216 are within the hypothetical binding site. Arg94 was introduced in round 2 of the affinity maturation and Arg167 was an arginine insertion in round 3 of the affinity maturation. Arg31 is a mutation from the germline sequence that was within one of the two starting antibodies isolated in the lead isolation round. Arg162 and Arg166 are both residues that are within the germline sequence that contribute to the positive electrostatic potential in the binding site. In addition, Arg216, which is within V_HCDR3 and is not a mutation caused by the affinity maturation process, also contributes to the positive electrostatic potential. It is interesting to point out that two histidines were added during the affinity maturation and appear at the center of the binding site. His218 was introduced in round 2 of the affinity

maturation and His147 was introduced in round 3. His33 is a mutation from germline and is also within the binding site. Histidines may or may not be protonated at physiological pH depending on the local environment. Thus, it is possible that the histidine mutations may further contribute to positive charge within the binding site. One negatively charged group, Glu213, was mutated to Gly213 in round 1 of the affinity maturation thereby removing a negatively charged residue from the biding site. It appears that optimization of charge within the antigen binding site was vital for the affinity maturation of this antibody.

3.5 Discussion

Receptor revision and receptor editing (recombinatorial mutagenesis) both have been shown to occur during affinity maturation in human germinal centers[9, 10, 73]. B cell immunoglobulin gene rearrangements in the periphery have been theoretically described as a way to allow antibodies to make large leaps in sequence space in order to escape local optima on the affinity landscape[58]. This additional process of receptor diversification is believed to have an advantage over affinity driven selection based upon point mutations alone because point mutations allow for an antibody to affinity mature only toward a local optimum (Figure 3.7). Because the antibody must pass through lower affinity intermediates, which are lost during affinity driven selection, to reach other optima, the antibody cannot move to other regions of the affinity landscape that potentially contain optima of higher affinity. The immune system appears to exploit recombinatorial mutagenesis to swap in larger functional portions of the variable antibody domain, generating binding motifs with substantially altered structure and higher affinity than can be obtained by point mutation alone.

The previous explanation provides a theoretical basis for recombinatorial mutagenesis to exist *in vivo*. This logic can be extended to provide a basis for using recombinatorial mutagenesis as a strategy for creating antibody libraries *in vitro*. For example, the benefits of using recombinatorial mutagenesis in addition to point mutagenesis becomes clear when one considers the median fitness of a population and the frequency distribution of fitness in a population generated by each method[74]. A library generated by point mutagenesis of a given antibody will have a slightly lower median fitness than the fitness of the starting antibody. In addition, the fitness distribution will be narrow. A library generated by using recombinatorial mutagensis of a given antibody will exhibit a much lower median fitness than a point mutagenesis generated library but will have a much larger fitness distribution. As a result, most clones in the library will exhibit much lower fitness than the starting point but the large variance increases the likelihood that the best clones in a recombinatorial mutagenesis library will exhibit higher fitness than the best clones in the point mutagenesis library.

However, *in vitro* methods of generating library diversity other than point mutagenesis have not yielded remarkable results. First, there is no facile method for making libraries with amino acid insertions and deletions because the residues that need to be added to or removed are not readily apparent. Methods based upon receptor editing or receptor revision have been used but still have not been successful enough to become common practice. Large scale replacement of whole regions of scFv DNA were successful in engineering a binder against a hapten with an affinity improvement of 2 orders of magnitude[42] but had little success against a protein antigen[65]. Furthermore, in a study by Ellemark *et al.*, swapping of the V_HCDR2 and light chain did not yield anti-

protein antibodies with higher K_d values but did produce antibodies with faster on rates[66]. What becomes clear is that the success of a given mutagenesis tactic is an unpredictable consequence of the specific antibody lineage and antigen – thus, the capability to rapidly explore multiple mutagenic mechanisms raises the probability of generating a functionally improved mutant antibody.

In this paper, we have successfully employed an integrated approach utilizing both random mutagenesis and recombinational mutagenesis to affinity mature an anti-peptide scFv at least five orders of magnitude. This approach incorporated random mutagenesis in each round of affinity maturation but also included beneficial receptor editing in round 1 and receptor revision plus amino acid insertion in round 3. In round 3, where receptor revision and random mutagenesis were compared head to head, each method generated equivalent affinity improvements although the recombinatorial mutagenesis library was 77 fold smaller than the random mutagenesis library. However, this result does refute the hypothesis that recombinatorial mutagenesis allows for greater improvements in affinity than point mutagenesis. First, the libraries compared were not of equal size. Second, it is possible that several local optima on the affinity landscape correspond to roughly equivalent affinities. Therefore, it is possible that the local optimum that the antibody reached by random point mutagenesis in round 3 was of the same affinity as the local optimum in a different place on the affinity landscape that the antibody was able to find through recombinatorial mutagenesis. In addition, although not applicable to the case considered in this paper, it is important to mention that if an antibody is at an affinity near the global optimum, recombinatorial mutagenesis can only lead to reduced fitness. Likewise, if an antibody is at its local optimum, recombinatorial

mutagenesis is the only method that can lead to increased fitness. Therefore, the success of a given method is highly dependent on the location of a given antibody on the affinity landscape. In order to fully compare the two methods, several antibodies must be affinity matured against different antigens using libraries of equivalent size.

The results of this paper, however, do validate recombinatorial mutagenesis as a mutational strategy and demonstrate at which step in the affinity maturation process receptor editing and receptor revision may be beneficial. There is evidence that receptor editing is more likely to be beneficial when the affinity for the antigen is low, probably in the hundreds of nanomolar range or weaker. This observation is in agreement with Marks *et al.* in which an anti-hapten antibody (K_d = 320 nM) was first affinity improved by shuffling the light chain and then further improved in a receptor revision like event yielding a total of 2 orders of magnitude affinity improvement[42]. When receptor editing and receptor revision were performed separately on an antibody that already had a 16 nM affinity for a protein antigen, improvements achieved by each method were not additive[65]. As Tomlinson *et al.* demonstrated, diversity in the antibody primary repertoire is confined to the CDR3 of each chain and spreads outward to the periphery of the binding site during somatic hypermutation[61]. It may be that in a similar manner receptor revision makes changes in the V_HCDR1 and V_HCDR2, which are part of the binding site periphery, to allow for affinity improvements after some threshold affinity has been reached which is determined by the CDR3s of the heavy and light chains. This explanation is consistent with our observation that receptor editing was beneficial in round 1 when the affinity of the parental pool was low but did not yield an affinity improvement in round 3 when the parental antibody already had nanomolar affinity.

Receptor revision, however, was able to generate a two orders of magnitude affinity improvement in round 3 despite the relatively high affinity of the parental antibody.

Moreover, a facile method for recombinatorial mutagenesis that is not possible using display technologies other than yeast is demonstrated. The high fidelity method of *in vivo* homologous recombination in yeast allows for the creation of hybrid genes between a known sequence and nonimmune library DNA without PCR assembly, ligation, and cloning. Thus, recombinatorial mutagenesis is as easy to use as random mutagenesis. Furthermore, it is the only known method that allows for easy access to beneficial amino acid insertions and deletions that have been retained in the starting non-immune antibody repertoire.

Our results suggest that it is best to incorporate multiple mutagenic strategies in the affinity maturation. Chain shuffling should be used in the early rounds of the affinity maturation but not when the affinity has improved to the nanomolar range. Although in the case shown here, recombinatorial mutagensis lead to an affinity improvement equal to that obtained through random mutagensis, recombinatorial mutagenesis, because it allows for the possibility of significant structural change, has the advantage of potentially creating antibodies that may have reduced framework divergence, different cross reactivity, and different therapeutic potential. The strategy presented here is uniquely capable of straightforwardly mimicking the genetic spectrum generated by B cells during affinity maturation *in vivo*. Our integrated approach, made possible by the *in vivo* homologous recombination mechanism in yeast, should prove a valuable tool for antibody engineering.

Table 3.1: Amino acid sequences of the CDRs of the best clones isolated in each round of affinity maturation for anti-PSGL-1 antibodies.

A. Round 0

Clone Name	V_HCDR1	V_HCDR2	V_HCDR3	V_LCDR1	V_LCDR2	V_LCDR3	Other Mutations
r0s3-6	SNNVAWN	RTYYRSKWYNDYAASVKS	EGGRAQSA	RASQRVSTTYLA	AASRRAT	QHYRSSPPLT	
r0s3-15	SYYWT	YIHNSGSTNYNPSLKS	RLKSGWFAGWFGP	RASQSVSRSHLA	GASSRAT	QQYGSPGVT	

B. Round 1

Clone Name	V_HCDR1	V_HCDR2	V_HCDR3	V_LCDR1	V_LCDR2	V_LCDR3	Other Mutations
r1s6-15	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAQSA	RASQSVSRSHLA	GASSRAT	QQYGSPGVT	

C. Round 2

Clone Name	V_HCDR1	V_HCDR2	V_HCDR3	V_LCDR1	V_LCDR2	V_LCDR3	Other Mutations
r2s4-2	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA	RASQSVSRSHLA	GASSRAT	QQYGRPGVT	Linker G to R

D. Round 3

Clone Name	V_HCDR1	V_HCDR2	V_HCDR3	V_LCDR1	V_LCDR2	V_LCDR3	Other Mutations
r3s4-3	SNNVAWN	RTYYRSKWYNDYAASVKG	GGSRAHSA	RASQSVSRSHLA	GVSSRAT	QQYGRPGVT	
RR1r3s4-2	SNTAAWN	RTYYRRSKWYNDYAASVKS	GGGRAHSA	RASQSVSRSHLA	GASSRAT	QQYGRPGVT	V_HFR1 Q to H V_HFR1 V to I
RR2r3s4-1	SNAAAWH	RTYYRRSKWNYDYALSVKS	GGGRAHSA	RASQSVSRSHLA	GASSRAT	QQYGRPGVT	V_HFR3 T to N V_HFR3 A to P V_HFR3 Q to L V_HFR3 H to Q

Table 3.2: CDR amino acid sequences for a random sampling of clones from round 3 receptor revision and receptor editing libraries directed against PSGL-1 prior to sorting.

A. Round 3: Receptor Revision with V_HFR1 + V_HCDR1 Preferentially Exchanged

# of clones	V_HCDR1	V_HCDR2	V_HCDR3
original	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
4	SNSAAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	SNSATWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	GNSATWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	TNNTAWN	RTYYRSKWYNDSAVSMKS	DGGLGPQR
1	SNAAGWN	RTYYRSKWSTDYAVSVKG	ARWGGLERRLYHFDF
1	RFAVS	VIPMFGTPKYAQRFQG	DAARGYGSGIEAFDI

B. Round 3: Receptor Revision with V_HFR1, V_HCDR1, V_HFR2, V_HCDR2 Preferentially Exchanged*

# of clones	V_HCDR1	V_HCDR2	V_HCDR3
original	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	SNTAAWN	RTCYRSKWYFDYAPSVKS	GGGRAHSA
1	SNTAAWN	RTYYRSKWYNDYAVSVKS	GGGRAHSA
1	HDGAAWN	RTYYRSKWYYGYAISVKS	GGGRAHSA
1	SNTAAWN	RTYYRSKWYSDYAVSLKS	GGGRAHSA
1	NTDAAWH	RTNFRSKWSNDYALFVKS	QYGWNLGH
1	SNSAAWN	RTHYRFKWYSDYAVSVKS	GSKSSFDY
1	SNSAIWN	RTYYRSQWYNDYAVSVKS	DLAGFDY
1	DYAMH	GISWNSGSIAYADSVKG	DIRGYYDSSGGFDP

C. Round 3: Receptor Editing†

# of clones	V_LCDR1	V_LCDR2	V_LCDR3	Chain Name
original	RASQSVSRSHLA	GASSRAT	QQYGRPGVT	A27
1	RASQSVSSSYLA	DASNRAT	QQRSDWPRT	L6
1	RASQSVSSSYLA	SASSRAT	QQHGSSPYT	A27
1	TGTSSDVGGYNCVS	DVTKRPS	QSYDGSNHAV	V1-3
1	RTSQSIYRYLN	AVSSLQT	QQLKSYPRIT	O2

*Ten total clones were sequenced. Clones not shown contained only a light chain.

†Ten total clones were sequenced. Clones not shown contained only a light chain or had a duplicate heavy chain and a new light chain.

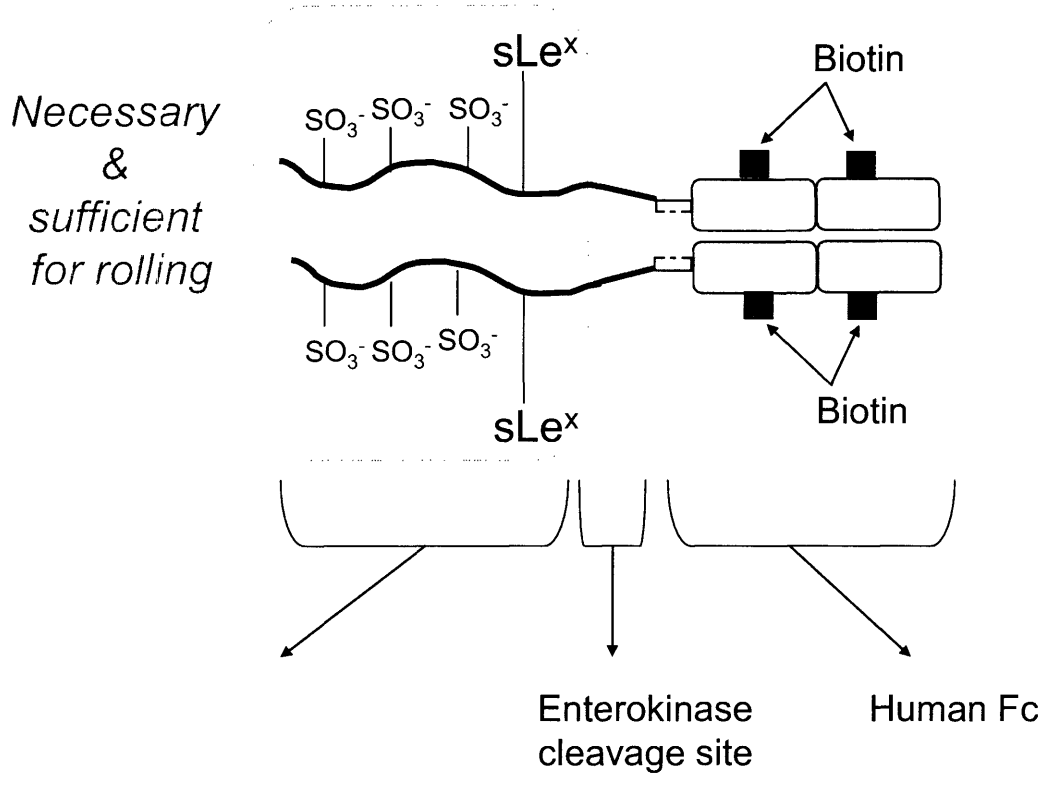


Figure 3.1: The b.19.ek.Fc construct. This construct contains the N-terminal nineteen amino acids of PSGL-1 that have been determined to be necessary and sufficient for rolling. These nineteen amino acids contain three sites of tyrosine sulfation and one site of O-linked glycosylation. Two of these peptides are fused to a human Fc. An enterokinase cleavage site allows for the isolation of the monovalent peptide.

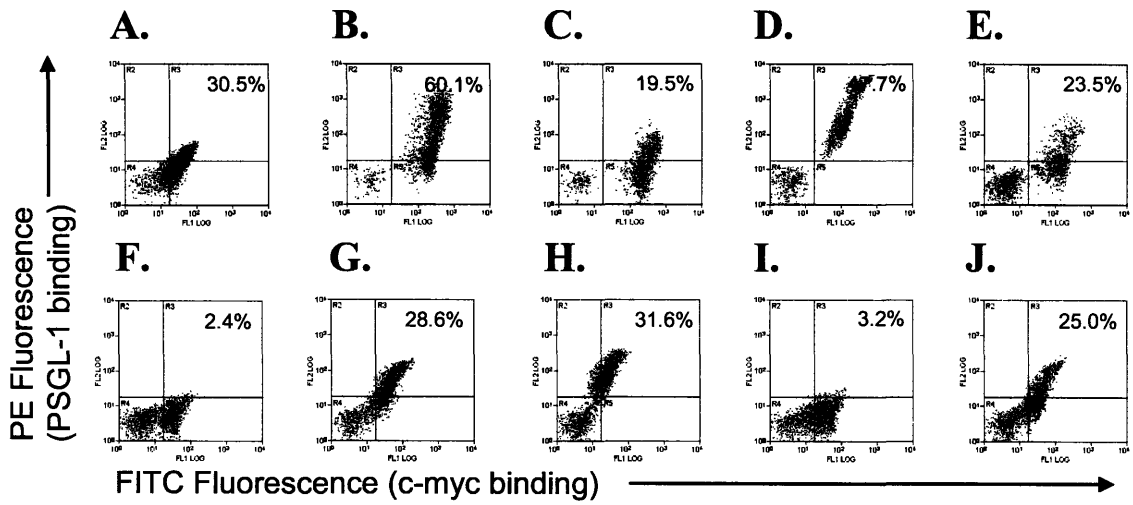
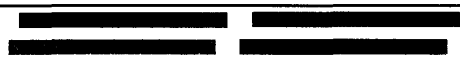


Figure 3.2: Bivariate histograms showing round by round affinity maturation. The numbers in the upper right corner of each histogram indicate the percentage of double positive clones. (A) Best clone from round 0, 800 nM divalent PSGL-1. (B) Best clone from round 1, 800 nM divalent PSGL-1. (C) Best clone from round 1, 50 nM divalent PSGL-1. (D) Best clone from round 2, 50 nM divalent PSGL-1. (E) Best clone from round 2, 50 nM monovalent PSGL-1. (F) Best clone from round 2, 0.5 nM monovalent PSGL-1. (G) Best clone from round 3 RR1 library, 0.5 nM monovalent PSGL-1. (H) Best clone from round 3 RR2 library, 0.5 nM monovalent PSGL-1. (I) Best clone from round 3 receptor editing library, 0.5 nM monovlanet PSGL-1. (J) Best clone from round 3 mutagenic library, 0.5 nM monovlanet PSGL-1.

Round	Schematic Representation	K_d Divalent PSGL-1	K_d Monovalent PSGL-1
Round 0			
Lead Isolation		> 5 μM	> 12 μM
Round 1			
Receptor Editing + Random Mutagenesis		1- 5 μM	5 – 12 μM
Round 2			
Random Mutagenesis		90 ± 110 pM	50 ± 30 nM
Round 3			
Random Mutagenesis		not determined	180 ± 100 pM
Receptor Revision with V_HFR1 + V_HCDR1 Preferentially Exchanged		not determined	220 ± 200 pM
Receptor Revision with V_HFR1, V_HCDR1, V_HFR2, V_HCDR2 Preferentially Exchanged		not determined	70 ± 60 pM
Receptor Editing		no improvement over previous round	

* Indicates an insertion mutation, black bars indicate point mutations.

Figure 3.3: Round by round schematic of affinity maturation methodology with best clone K_d values for anti-PSGL-1 antibodies. A pool of antibodies was isolated in Round 0 and affinity matured over at least five orders of magnitude in three library construction and screening steps to lead to the generation of three different subnanomolar binders as measured by titration with monovalent PSGL-1 on the surface of yeast.

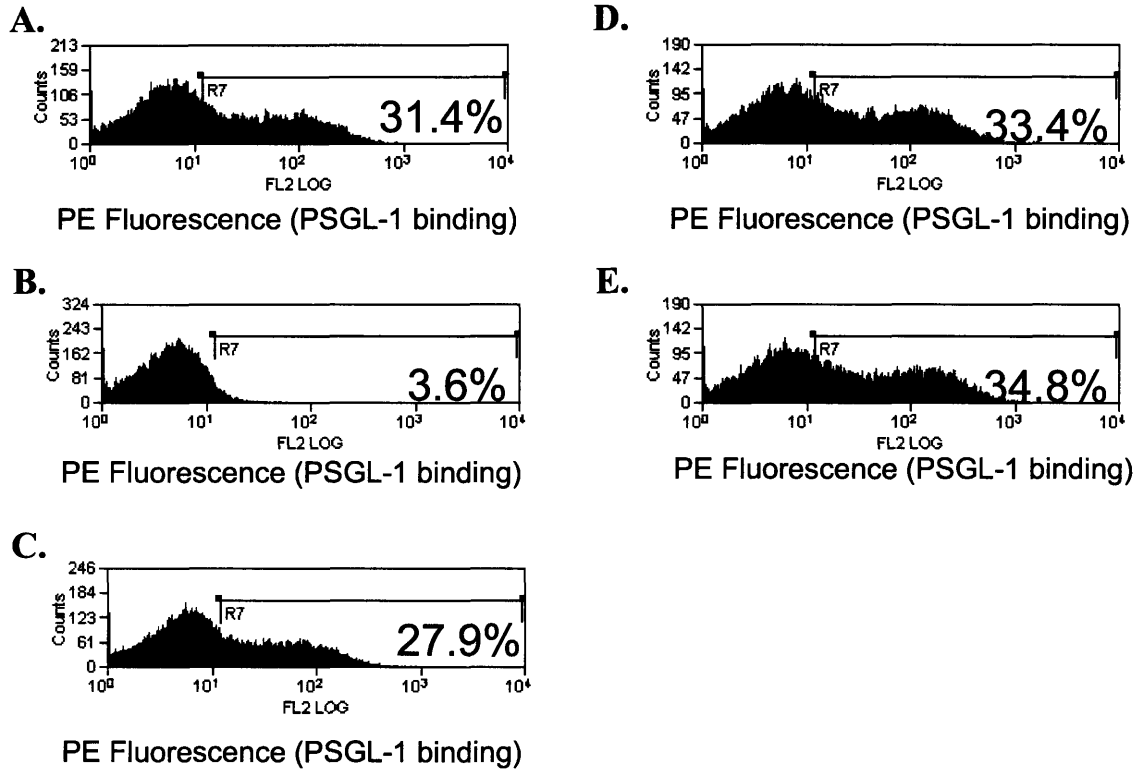


Figure 3.4: Histograms demonstrating the specificity of r1s6-15. Percentages refer to the percent of clones within the region specified on each histogram. (A) Binding of r1s6-15 to 50nM of b.19.ek.Fc. (B) Decreased binding of r1s6-15 to 50 nM of b.19.ek.Fc in the presence of a 10 fold excess of KPL1. (C) Binding of r1s6-15 to 50nM of b.19.ek.Fc in the presence of 500 nM of an anti-enterokinase monoclonal antibody. (D) Binding of r1s6-15 to 100nM of b.19.ek.Fc. (E) Binding of r1s6-15 to 100 nM of b.19.ek.Fc in the presence of a 30 fold excess of human Fc.

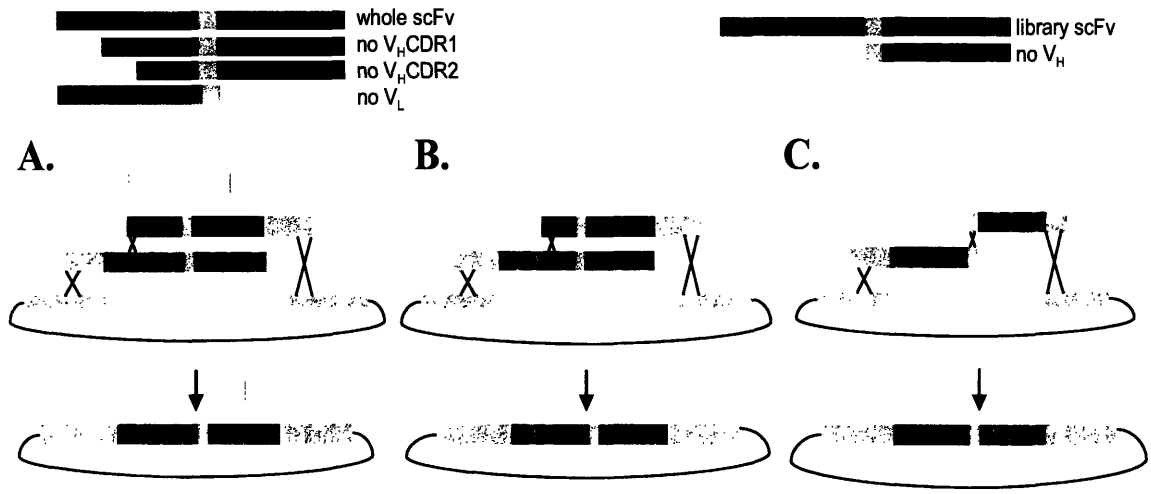


Figure 3.5: Schematic representation of mutagenesis strategies. (A) V_HFR1 + V_HCDR1 are preferentially exchanged (RR1 library). (B) V_HFR1, V_HCDR1, V_HFR2, V_HCDR2 are preferentially exchanged (RR2 library). (C) Shuffling of the light chains (RE library). The red and pink regions represented the antibody CDRs while the blue and brown regions represented the antibody framework regions. The light green region in the center of each PCR fragment is the $(gly_4ser)_3$ linker. The gray regions on the 5' or 3' end of the PCR fragments indicate homology to the cut yeast surface display vector.

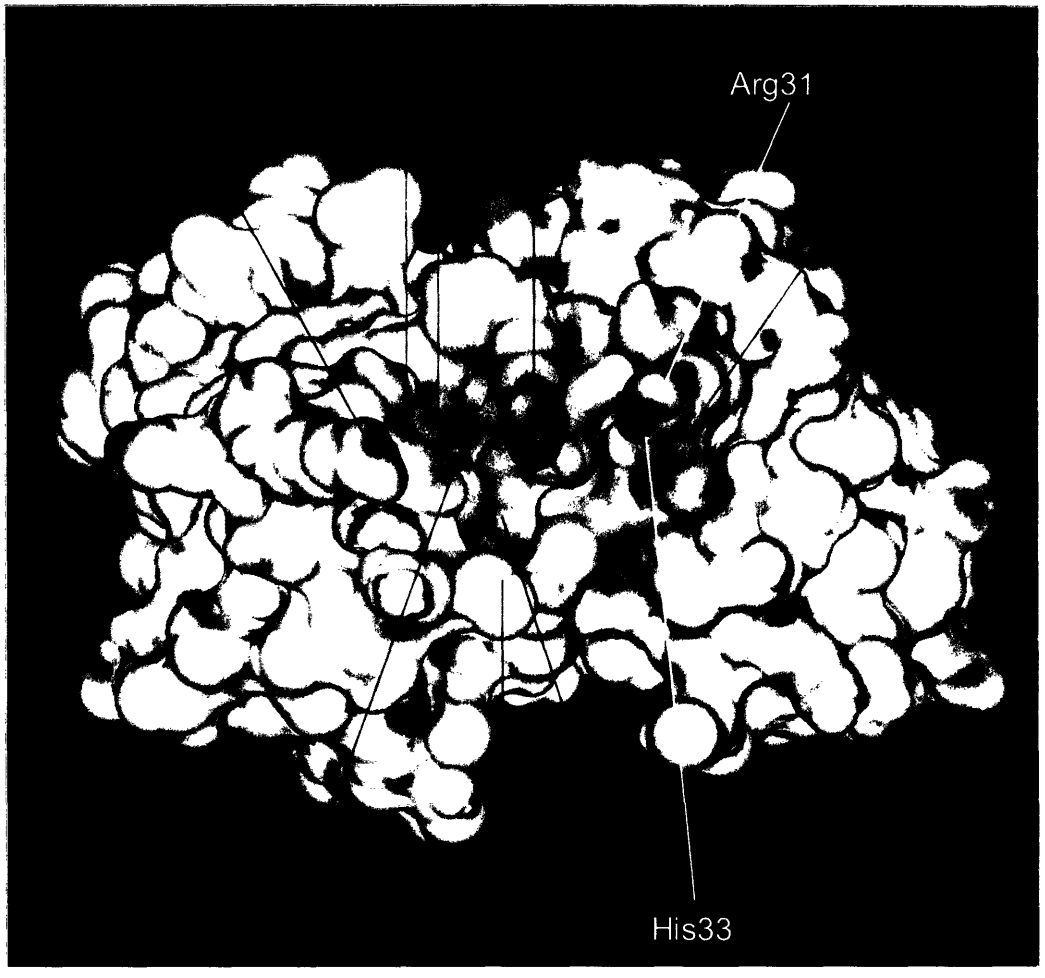


Figure 3.6: Representation of the electrostatic potential at the surface of clone RR2r3s4-1 (top-down view). The heavy chain is shown on the left and the light chain is shown on the right. Blue indicates regions of positive charge and red indicates regions of negative charge. Residues referred to in purple text are mutations introduced during affinity maturation. Residues referred to in yellow text are germline mutations that were present in the starting antibodies prior to affinity maturation. Residues referred to in green text are residues that are positively charged but are not germline mutations or mutations introduced during the affinity maturation.

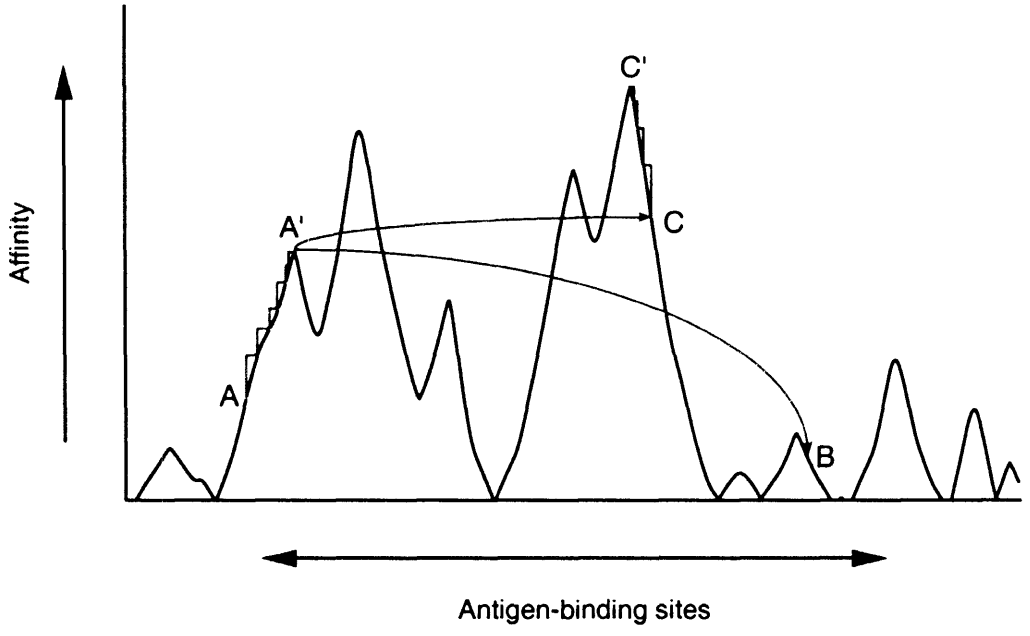


Figure 3.7: Theoretical representation of the movements in sequence space caused by receptor revision (reprinted from George and Gray).[58] The x-axis shows all possible binding sites with the most similar adjacent to one another. The affinity for a given antigen is shown on the y-axis. An antibody starting at point A can only move toward the local optimum point A' through point mutation. Receptor revision allows for large jumps in sequence space that are usually detrimental (a movement to point B on the graph) but sometimes lead to a new location (point C) that is nearer to a local optimum of higher affinity (point C') than the optimum closest to the starting point.

Chapter 4: Characterization of a Novel High Affinity Antibody Against PSGL-1

4.1 Introduction

We have developed a high affinity, fully human IgG directed against the N-terminal nineteen 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 the initial rolling of leukocytes on the lumenal surfaces of the vascular endothelium[75]. Although many glycoproteins have been shown to support rolling *in vitro*, PSGL-1 is the only glycoprotein to be identified that has a clearly defined role as a physiological ligand for P-selectin[23, 28]. The N-terminal nineteen amino acids of PSGL-1 have been shown to be both necessary and sufficient to mediate attachment and rolling on P-selectin *in vivo*[24]. 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[76]. 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[23].

Blocking of P-selecton-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% ± 7% two hours after stimulation and 59% ± 7.9%

four hours after stimulation[77]. 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 IgG1 Fc has been generated as a therapeutic by Wyeth and has proven to be therapeutically effective in animal trials. For example, rPSGL-1-Ig has been shown to accelerate thrombolysis and prevent reocclusion in a porcine model[78], to attenuate leukocyte-endothelial interactions after hemorrhagic shock in mice[79], and to protect against ischemia/reperfusion injury in cats[80].

The disadvantage of rPSGL-1-Ig is its low affinity which is equal to that of PSGL-1 for P-selectin (K_d = 320 ± 20 nM)[26]. An alternative method of blocking P-selectin-PSGL-1 interactions is the use of monoclonal antibodies which can have their affinity increased 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 mouse derived monoclonal antibodies directed against PSGL-1 such as KPL1, PL1 and PL2, are known[71, 81], they are not suitable as therapeutics because of immunogenicity issues. Currently, only one human monoclonal against PSGL-1 has been published[82]. This antibody, which is property of Wyeth, does not have a published K_d.

In this paper we characterize the binding properties of a high affinity IgG, called RR2r3s4-1, and a weaker affinity parent IgG, called r2s4-2. Both of these IgGs were converted from scFvs (Figure 4.1) that had been engineered to high affinity from a pool of anti-PSGL-1 binders (see Chapter 3) isolated from a yeast surface displayed non-immune library[17]. The K_ds were measured using tryptophan quenching titration. The antibody RR2r3s4-1 was verified to bind neutrophils by flow cytometry. In addition,

these antibodies were compared to the mouse monoclonal antibody KPL1 in both an ELISA and a neutrophil rolling inhibition assay using a parallel wall flow chamber. To our knowledge, RR2r3s4-1 is the most effective antibody at inhibiting P-selectin-PSGL-1 interactions known.

4.2 Materials and Methods

4.2.1 IgG Production

The human IgGs used in this paper contain the variable heavy and variable light regions from scFvs that have been described previously. The variable heavy and variable light chain DNA was cloned into an IgG_1 heavy chain vector and a κ light chain vector both generously provided by Wyeth (Cambridge, MA). IgGs were produced in T 175 flasks using COS cells (Wyeth, Cambridge, MA). For each T175, COS cells were grown in 30 mls of DME plus 10% heat inactivated fetal bovine serum (Sigma, St. Louis, MO) plus 1mM glutamine plus 100 IU/ml penicillin (Mediatech, Herndon, VA) plus 100 μg/ml streptomycin (Mediatech, Herndon, VA). Trypsin was used to make the adherent COS cells soluble in the growth media each time cells were spilt. Cells were split 1 to 2, grown for three days, then split 1 to 3 and grown for two days. After the growth phase cells were transfected with DNA using the follwing protocol. For each T175 flask, 7.0 ml of Opti-MEM media supplemented with glutamine was mixed with 125 μl of TransIT (Mirus, Madison, WI), vortexed and incubated for 15 minutes at room temperature. While incubating, 25 μg of heavy chain and 25 μg light chain DNA were mixed. After incubation the DNA was added to the Opti-MEM (Invitrogen, Carlsbad, CA) plus TransIT, mixed gently by pipeting, and incubated for 15 minutes at room temperature.

This mixture was added slowly dropwise to the T175 containing the COS cells and growth media. The next day the cells were rinsed twice with sterile PBS and 30 ml of serum-free media (Wyeth, Cambridge, MA) media plus 1mM glutamine plus 100 IU/ml penicillin plus 100 μg/ml streptomycin was added. After 48 hours the first harvest was collected, spun in 250 ml conical bottles, and filtered through a 0.2 μM filter. Another 30 ml of serum-free media plus glutamine plus penicillin plus streptomycin was added and 72 hours later the same harvesting procedure was repeated.

4.2.2 IgG Purification

An HPLC (Millipore, Billerica, MA) was used to load a recombinant protein A HiTrap FF 1 ml column (Amersham Biosciences, Piscataway, NJ). Before loading, the harvests were pooled and 1/10 volume 5M NaCl was added and the sample was filtered. Loading was done at 4°C overnight. After loading the column, 10 ml of PBS was run through the column. The elution was performed on an FPLC (Amersham Biosciences, Piscataway, NJ) and the sample was concentrated and buffer exchanged into PBS using a Centriprep YM-10 (Millipore, Billerica, MA). The concentration was determined by taking the OD_{280} using an extinction coefficient of 1 OD_{280} = 1.4 mg/ml.

4.2.3 Tryptophan Fluorescence Quenching Titration

K_ds were determined using an Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA). Between each measurement, the 3 ml quartz cuvette was thoroughly cleaned with 5M HNO_3. Antibodies were diluted into PBS at a concentration of 20 nM. The ligand used was a monovalent form of 19.ek.Fc called 19.ek (which was a kind gift from Gray Shaw, Wyeth, Cambridge, MA) which lacks the Fc portion but does contain the N-terminal 19 amino acids of PSGL-1. 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 cuvette was rotated end over end five times to ensure proper mixing. The excitation and emission wavelengths were 290 nm and 340 nm, respectively, and the excitation and emission slits were both set to 5 nm. The PMT voltage was set to high.

4.2.4 Inhibition ELISA

A clear 96-well ELISA plate (Corning, Acton, MA) was coated with human P-selectin (which was a kind gift from Diane Sako, Wyeth, Cambridge, MA) at a concentration of 1 μg/ml, 100 μl per well, overnight at 4°C. The buffer for coating consisted of 10 mM MOPS, 150 mM NaCl, 1 mM $CaCl_2$, 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). Blocking was performed with 200 μl blocking buffer per well, shaking, at room temperature for one hour. In a separate, U-bottom, polypropylene plate called dilution plate 1, a standard curve was made in duplicate by diluting a starting concentration of 1 μg/ml (4X final concentration) of a biotinylated form of 19.ek.Fc, called b.19.ek.Fc (a gift of Dr. Raymond T. Camphausen, formerly of Wyeth, Cambridge, MA), serially 1 to 3 leaving 60 μl per well. The 19.ek.Fc protein contains the 19 N-terminal amino acids of PSGL-1 and has been described previously[24]. Column three of the plate received 60 μl per well diluent buffer. For the remaining columns, 60 μl per well of b.19.ek.Fc were aliquoted at 80 ng/ml (4X final conentration). To all wells on dilution plate 1, 60 μl of a 1 to 500 dilution of streptavidin-HRP (Pierce, Rockford, IL) was added. Dilution plate 1

was incubated at room temperature for thirty minutes in order to pre-complex the b.19.ek.Fc to the streptavidin-HRP.

To the first four columns of a U-bottom, polypropylene plate called dilution plate 2, 80 μl of diluent buffer were added. Antibodies were started in duplicate 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 were transferred from dilution plate 1 to dilution plate 2 and incubated at room temperature for fifteen minutes. After this incubation, 100 μl per well were transferred to the blocked P-selectin coated plate and incubated at room temperature for thirty minutes. The plate was then washed five times and 100 μl of pre-warmed TMB (BioFx, Owings Mills, MD) was added to each well and incubated at room temperature for between ten and fifteen minutes. To stop the reaction, 100 μl of 0.18 M H_2SO_4 was added to each well. The absorbance was read on a Molecular Devices UVMax plate reader at 450 nm.

4.2.5 Fluorescence Staining for Flow Cytometry

Neutrophils were isolated in the following manner. Whole blood was mixed 4 to 1 with 4.5% dextran in 0.9% NaCl. The mixture was left to settle for 90 minutes at room temp. The top clarified fraction was collected and washed in sterile PBS. After spinning, the pellet was resuspended in the small fraction of liquid left over after pouring off the PBS. Red blood cells were lysed by adding 9 mls of water, letting the cells sit in the water for 1 to 2 minutes, then adding 1 ml of 10X Hanks' balanced salt solution. Next, the cells were washed with PB then resuspended in PBS and counted using a

hemocytometer. Cells were analyzed within 1 to 2 hours of the completion of the neutrophil isolation.

Approximately 250000 cells were labeled. PBS plus 0.1% BSA was used as the dilution and wash buffer. The primary antibodies were biotinylated using an EZ-Link sulfo-NHS biotin (Pierce, Rockford, IL). Primary labeling was performed on ice for 30 minutes 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 to 200 for 30 minutes on ice. The cells were then washed and were suspended in 180 μl of PBS plus 0.1% BSA plus 20 μl of 7-amino-actinomycin-D (7AAD) (BD Pharmingen, San Diego, CA) and incubated for 15 minutes on ice. Windows were drawn using the 7AAD to gate out dead cells. Analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA).

4.2.6 Rolling Inhibition Assay

Polysterene 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 hours, washed with PBS, pH 9, blocked with PBS/2% human serum albumin (HSA) at 4°C for 2 hours, 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[83] 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 x 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 x 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 10X-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).

4.3 Results

4.3.1 Determination of Dissociation Constant (K_d)

The dissociation constant for r2s4-2 and RR2r3s4-1 were determined by tryptophan fluorescence quenching titration. This method involves the measurement of the decrease in fluorescence of a known quantity of antibody (in this case 20 nM) by the sequential addition of a given quantity of antigen (5 nM per datapoint). The following equation can be used to fit the data:

$$F = F_E - \left[(E_T + L_T + K_d) - \sqrt{(E_T + L_T + K_d)^2 - 4E_T L_T} \right] \times \frac{F_E - F_{EL}}{2E_T} \qquad \textbf{4.1}$$

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[84].

For each clone three replicates were performed. Using this method, the best fit K_d for r2s4-2 is 6.2 ± 3.0 nM and the best fit K_d for RR2r3s4-1 is 1.8 ± 0.7 nM (examples of one curve fit for each clone are shown in Figure 4.2). These K_ds differ from those

determined in Chapter 3. The K_d for r2s4-2 is about seven fold stronger and the K_d for RR2r3s4-1 is about twenty-seven fold weaker. This difference is due to the reformatting and may be because the constant regions of the IgG orient the domains in the variable region slightly differently than they are oriented in an scFv format.

4.3.2 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 nineteen amino acids of PSGL-1. It is important to demonstrate that this antibody binds to the physiologically relevant protein which is PSGL-1 expressed on the surface of human neutrophils. Figure 4.3 shows that 67 nM of biotinylated RR2r3s4-1 binds to human neutrophils while a biotinylated, isotype matched negative control antibody does not.

4.3.3 Inhibition ELISA

One way to compare the blocking effectiveness of the two antibodies presented in this paper with the previously characterized antibody KPL1 is to demonstrate each antibody's ability to inhibit the binding of b.19.ek.Fc to a P-selectin coated ELISA plate. The basic setup of this experiment is shown in Figure 4.4A. To determine the optimum concentration of b.19.ef.Fc for inhibition, a standard curve was made by serially diluting b.19ek.Fc that has been pre-complexed with strep-HRP. In order to determine binding inhibition, the signal for binding of the b.19.ek.Fc to P-selectin in the absence of antibody must not be too high or the detector will be saturated and must not be too low or the differences in signal caused by inhibition will be too small to measure. The curve shown in Figure 4.4B indicates why the optimal labeling concentration of b.19.ek.Fc is approximately 20 nM. This concentration is near the saturation limit of the detector but

does not exceed this limit thus differences in b.19.ek.Fc binding to P-selectin can be measured. The inhibition constants can be determined by fitting the data to the following equation:

$$Y = B_{\max}\left(1-\left(\frac{x^n}{K^n + x^n}\right)\right) + Y_2 \qquad \textbf{4.2}$$

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 IC50.

Figure 4.4C shows the results. RR2r3s4-1 has the lowest IC50, and thus is a better inhibitor of P-selectin adhesion to PSGL-1 than KPL1. As expected, r2s4-2 has the largest IC50 and therefore is the poorest inhibitor. It is important to mention that the actual value of the IC50s 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.

4.3.4 Rolling Inhibition

The ability of various antibodies to inhibit neutrophil rolling is shown in Figure 4.5. 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 three seconds after initial tethering." [85]

As Figure 4.5 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 control antibodies named "negative" and PL2 barely inhibit rolling at all at the highest concentration tested, 66.7nM. The "negative" antibody is an isotype matched antibody and its non-binding indicates that the binding site of the antibodies that do inhibit is necessary for inhibition. PL2 is an antibody that binds PSGL-1 but does not bind in the site that is necessary and sufficient for neutrophil rolling and was added as an addition control. The PL2 data is important because it shows that it is possible to bind to the neutrophils but not block rolling. Only antibodies that bind in the terminal nineteen amino acids will have the desired inhibitory effect. The rolling inhibition data demonstrates that RR2r3s4-1 is the best antibody at inhibiting neutrophil rolling that has yet been characterized. This data, in conjunction with the flow cytometry data, indicates that RR2r3s4-1 binds to PSGL-1 and this binding is within the epitope that is necessary and sufficient for neutrophil rolling.

4.4 Discussion

The paper demonstrates that a novel antibody, RR2r3s4-1, that has been isolated form a yeast surface displayed non-immune human library and engineered for high affinity binding to PSGL-1, has therapeutic potential. It binds to the correct cell type and blocks these cells from rolling in an *in vitro* flow assay. This inhibition is better than the

commercially available mouse monoclonal KPL1. Two assays, the rolling assay and an inhibition ELISA, demonstrate that this antibody inhibits binding of P-selectin to PSGL-1 better than KPL1. The only other human antibody, owned by Wyeth, does not have published characterization information therefore until this information is published, RR2r3a4-1 is the best inhibitor of P-selectin/PSGL-1 binding known.

The next step is to perform a tissue cross reactivity assay in order to determine the specificity of RR2r3s4-1. If the antibody does not show significant cross reactivity, animal studies must be performed to see if there is a therapeutic effect. Hopefully, this antibody will become a major weapon in the ongoing fight against acute and chronic inflammatory conditions.

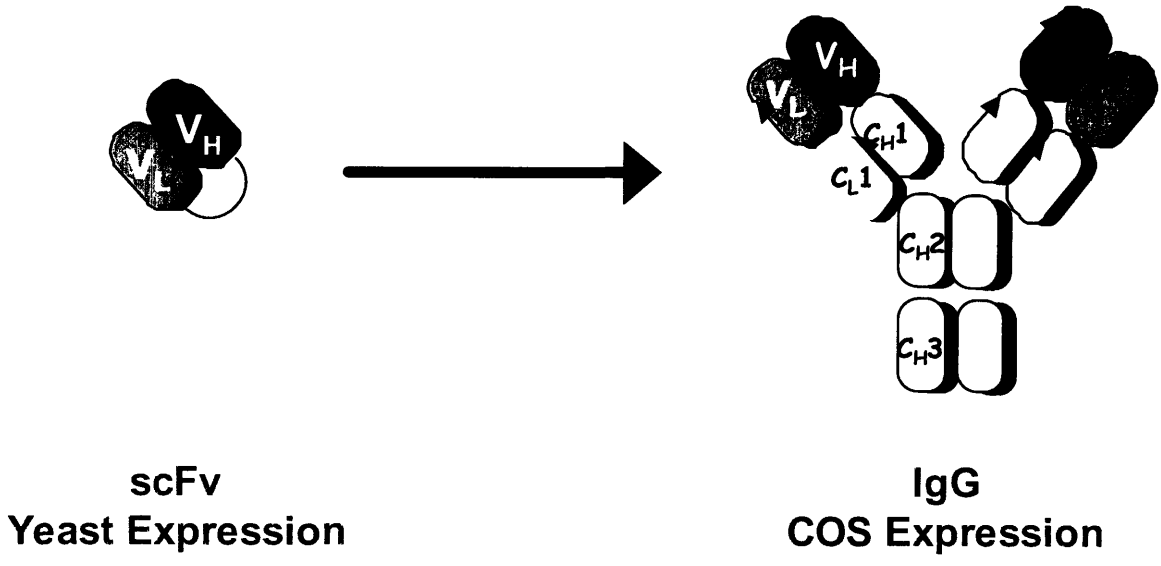
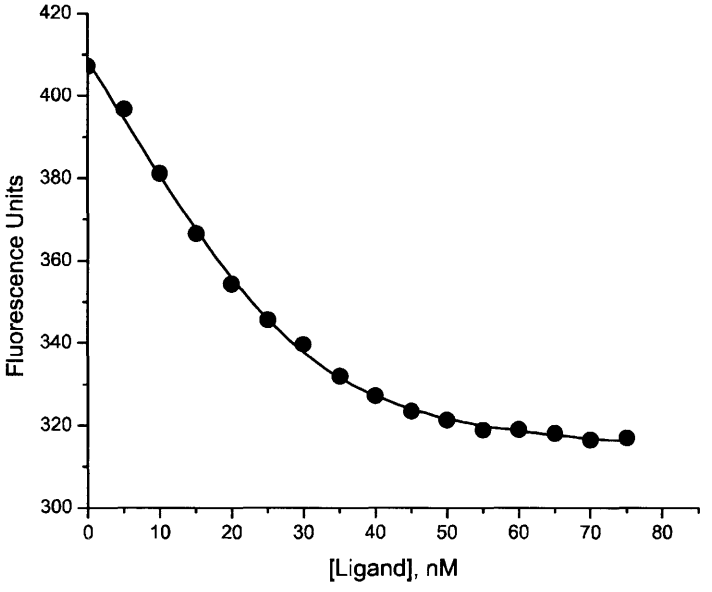


Figure 4.1: Conversion of an scFv to an IgG. The svFv contains only the variable region of an IgG and is monovalent. In an IgG, two identical variable regions are present making the molecule divalent. In this chapter, the IgGs are produced in COS cells, which are a mammalian cell line. This production system is different from the yeast expression system used to make the scFvs described in Chapter 3.

A.



B.

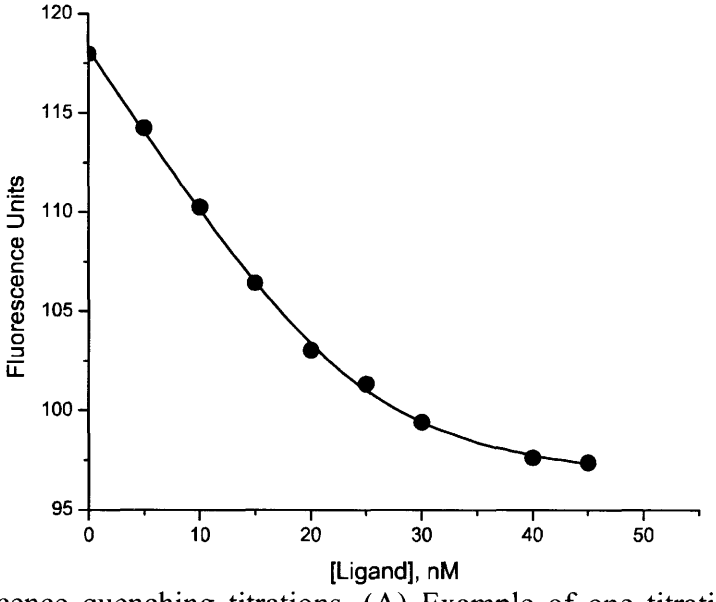


Figure 4.2: Fluorescence quenching titrations. (A) Example of one titration of clone r2s4-2. (B) Example of one titration of clone RR2r3s4-1.

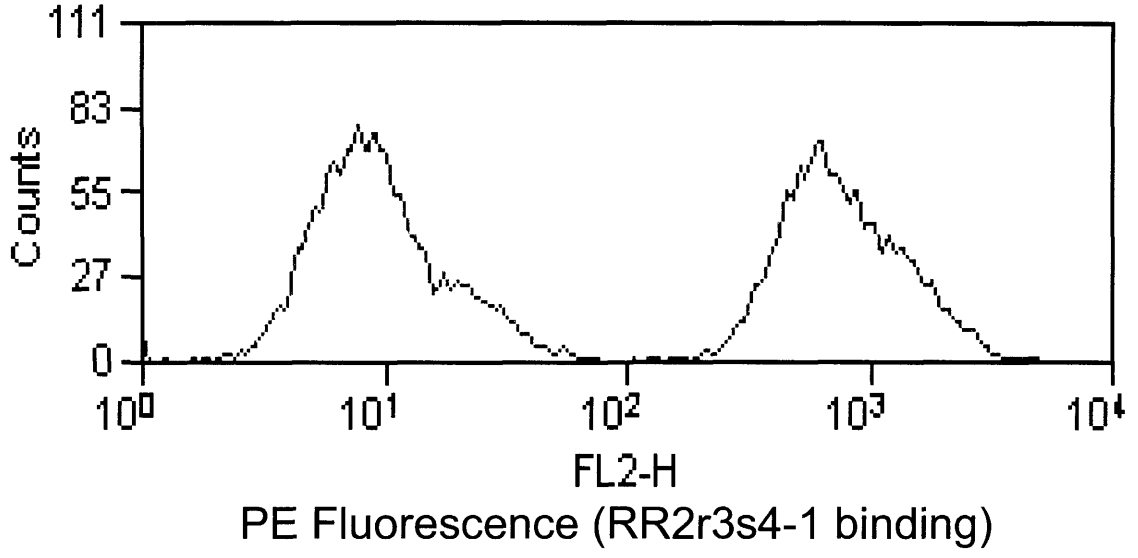


Figure 4.3: Binding of RR2r3s4-1 to human neutrophils. The red line indicates the histogram of the binding of 67 nM of biotinylated RR2r3s4-1 to human neutrophils. The blue line indicates the lack of binding of 67 nM of an isotype matched negative control antibody.

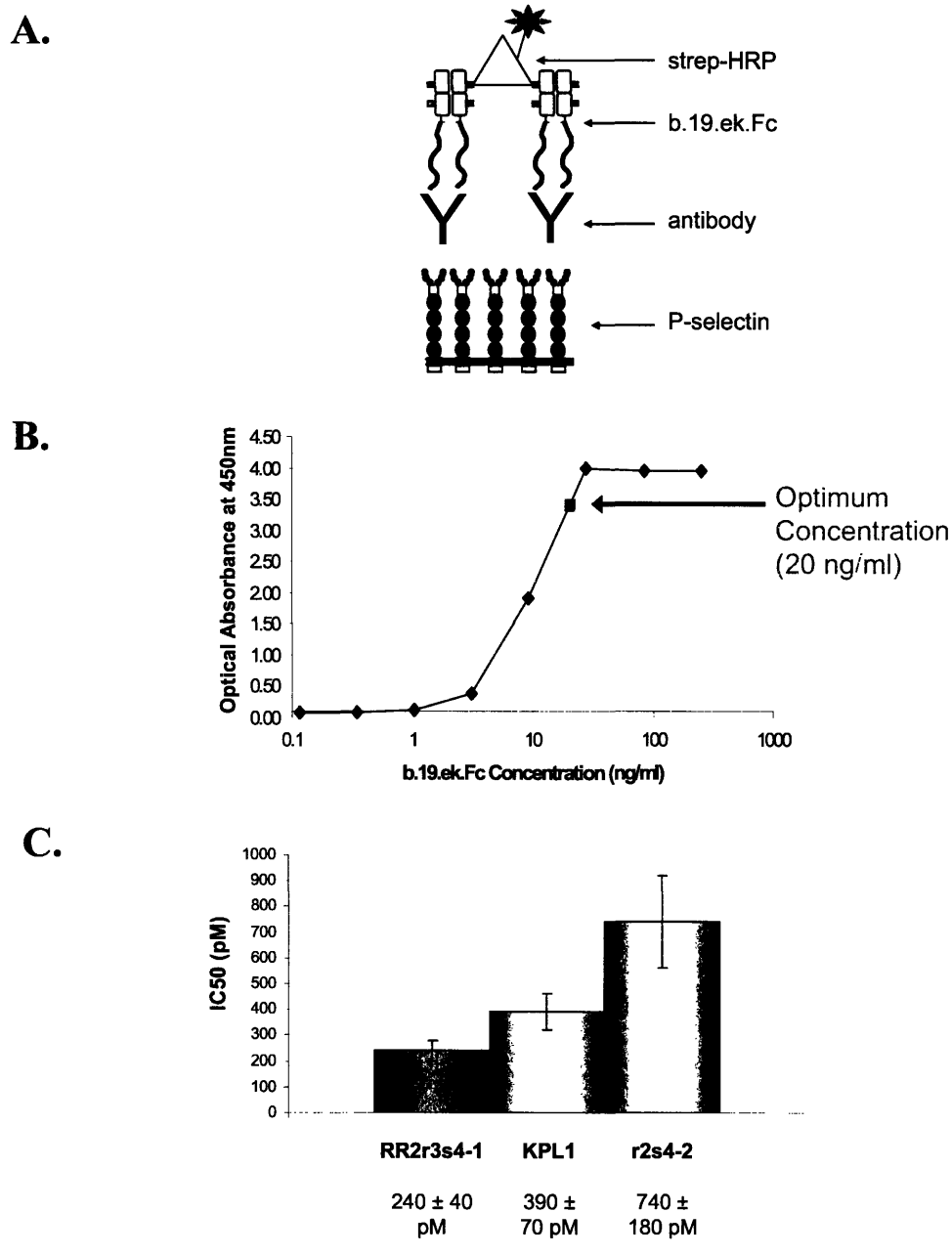


Figure 4.4: Inhibition ELISA. (A) Schematic of the inhibition ELISA setup. Strep-HRP is pre-complexed with b.19.ek.Fc. This complex can be prevented from binding to a P-selectin coated ELISA well by the presence of anti-PSGL-1 antibodies. (B) The optimal concentration for inhibition can be determined 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 (IC50) for RR2r3s4-1, KPL1, and r2s4-2.

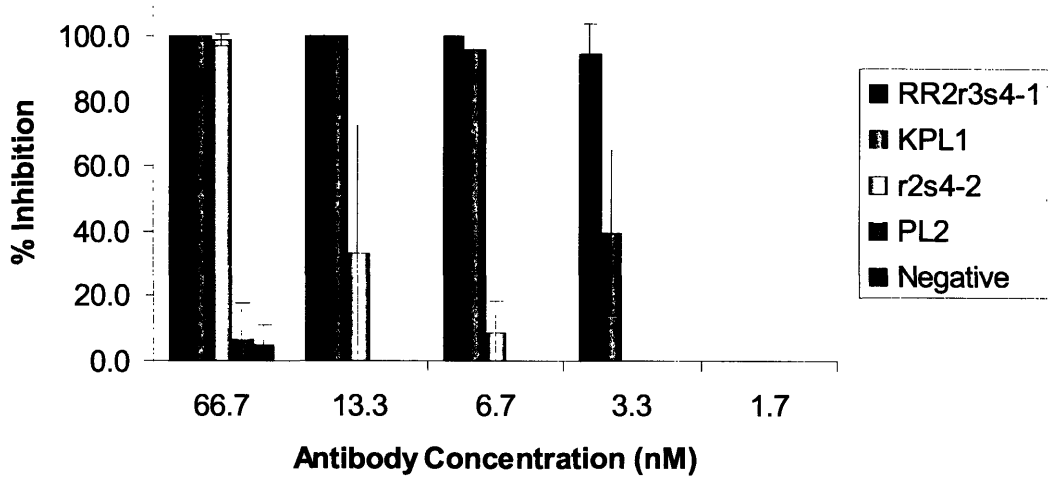


Figure 4.5: Percent of neutrophils inhibited from rolling by various antibodies. The antibody PL2 is a mouse monoclonal antibody that binds PSGL-1 but does not bind in the sight that is responsible for P-selectin binding. At the lowest concentration tested, 1.7 nM, none of the antibodies inhibited neutrophil rolling. The most potent rolling inhibitor was RR2r3s4-1 which blocked virtually 100% of cells rolling at a concentration of 3.3 nM.

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Appendix A: Amino Acid Sequence Divergence of Isolated Clones from Framework Sequence

r1s6-15 scFv Amino Acid Sequence

Sequence (red letters indicate divergence from framework sequence)	**Variable Chain Name**
V_HFR1	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	V_H6-01 FR1
V_HCDR1	
SNNVAWN	
SNSAAWN	V_H6-01 CDR1
V_HFR2	
WIRQSPSRGLEWLG	
WIRQSPSRGLEWLG	V_H6-01 FR2
V_HCDR2	
RTYYRSKWYNDYAASVKS	
RTYYRSKWYNDYAVSVKS	V_H6-01 CDR2
V_HFR3	
RITINADTSKNQFSLHLNSVTPEDTAVYYCTR	
RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	V_H6-01 FR3
V_HCDR3	
GGGRAQSA	
V_HFR4	
WGQGTLVTVSS	
$(Gly_4Ser)_3$ Linker	
GGGGSGGGGSGGGGS	
GGGGSGGGGSGGGGS	
V_LFR1	
EIVLTQSPGTLSVSPGERATLSC	
EIVLTQSPGTLSLSPGERATLSC	V_KIII 6-1-(1) A27 FR1
V_LCDR1	
RASQSVSRSHLA	
RASQSVSSSYLA	V_KIII 6-1-(1) A27 CDR1
V_LFR2	
WYQQKPGQAPRLLIF	
WYQQKPGQAPRLLIY	V_KIII 6-1-(1) A27 FR2
V_LCDR2	
GASSRAT	
GASSRAT	V_KIII 6-1-(1) A27 CDR2
V_LFR3	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	V_KIII 6-1-(1) A27 FR3
V_LCDR3	
QQYGSPGVT	
QQYGSSP	V_KIII 6-1-(1) A27 CDR3
V_LFR4	
FGQGTKVEIK	

r2s4-2 scFv Amino Acid Sequence

Sequence (red letters indicate divergence from framework sequence)	**Variable Chain Name**
V_HFR1	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	V_H6-01 FR1
V_HCDR1	
SNNVAWN	
SNSAAWN	V_H6-01 CDR1
V_HFR2	
WIRQSPSRGLEWLG	
WIRQSPSRGLEWLG	V_H6-01 FR2
V_HCDR2	
RTYYRSKWYNDYAASVKS	
RTYYRSKWYNDYAVSVKS	V_H6-01 CDR2
V_HFR3	
RITINADTSKNQFSLHLNSVTPEDTAVYYCTR	
RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	V_H6-01 FR3
V_HCDR3	
GGGRAHSA	
V_HFR4	
WGQGTLVTVSS	
$(Gly_4Ser)_3$ Linker	
GGGGSGGGGSRGGGS	
GGGGSGGGGSGGGGS	
V_LFR1	
EIVLTQSPGTLSVSPGERATLSC	
EIVLTQSPGTLSLSPGERATLSC	V_KIII 6-1-(1) A27 FR1
V_LCDR1	
RASQSVSRSHLA	
RASQSVSSSYLA	V_KIII 6-1-(1) A27 CDR1
V_LFR2	
WYQQKPGQAPRLLIF	
WYQQKPGQAPRLLIY	V_KIII 6-1-(1) A27 FR2
V_LCDR2	
GASSRAT	
GASSRAT	V_KIII 6-1-(1) A27 CDR2
V_LFR3	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	V_KIII 6-1-(1) A27 FR3
V_LCDR3	
QQYGRPGVT	
QQYGSSP	V_KIII 6-1-(1) A27 CDR3
V_LFR4	
FGQGTKVEIK	

r3s4-3 scFv Amino Acid Sequence

Sequence (red letters indicate divergence from framework sequence)	**Variable Chain Name**
V_HFR1	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	V_H6-01 FR1
V_HCDR1	
SNNVAWN	
SNSAAWN	V_H6-01 CDR1
V_HFR2	
WIRQSPSRGLEWLG	
WIRQSPSRGLEWLG	V_H6-01 FR2
V_HCDR2	
RTYYRSKWYNDYAASVKG	
RTYYRSKWYNDYAVSVKS	V_H6-01 CDR2
V_HFR3	
RITINADTSKNQFSLHLNSVTPEDTAVYYCTR	
RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	V_H6-01 FR3
V_HCDR3	
GGSRAHSA	
V_HFR4	
WGQGTLVTVSS	
$(Gly_4Ser)_3$ Linker	
GGGGSGGGGSRGGGS	
GGGGSGGGGSGGGGS	
V_LFR1	
EIVLTQSPGTLSVSPGERATLSC	
EIVLTQSPGTLSLSPGERATLSC	V_KIII 6-1-(1) A27 FR1
V_LCDR1	
RASQSVSRSHLA	
RASQSVSSSYLA	V_KIII 6-1-(1) A27 CDR1
V_LFR2	
WYQQKPGQAPRLLIF	
WYQQKPGQAPRLLIY	V_KIII 6-1-(1) A27 FR2
V_LCDR2	
GVSSRAT	
GASSRAT	V_KIII 6-1-(1) A27 CDR2
V_LFR3	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	V_KIII 6-1-(1) A27 FR3
V_LCDR3	
QQYGRPGVT	
QQYGSSP	V_KIII 6-1-(1) A27 CDR3
V_LFR4	
FGQGTKVEIK	

RR1r3s4-2 scFv Amino Acid Sequence

Sequence (red letters indicate divergence from framework sequence)	**Variable Chain Name**
V_HFR1	
QVHLQQSGPGLVKPSQTLSLTCAISGDSIS	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	V_H6-01 FR1
V_HCDR1	
SNTAAWN	
SNSAAWN	V_H6-01 CDR1
V_HFR2	
WIRQSPSRGLEWLG	
WIRQSPSRGLEWLG	V_H6-01 FR2
V_HCDR2	
RTYYRRSKWYNDYAASVKS	
RTYYRSKWYNDYAVSVKS	V_H6-01 CDR2
V_HFR3	
RITINADTSKNQFSLHLNSVTPEDTAVYYCTR	
RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	V_H6-01 FR3
V_HCDR3	
GGGRAHSA	
V_HFR4	
WGQGTLVTVSS	
$(Gly_4Ser)_3$ Linker	
GGGGSGGGGSRGGGS	
GGGGSGGGGSGGGGS	
V_LFR1	
EIVLTQSPGTLSVSPGERATLSC	
EIVLTQSPGTLSLSPGERATLSC	V_KIII 6-1-(1) A27 FR1
V_LCDR1	
RASQSVSRSHLA	
RASQSVSSSYLA	V_KIII 6-1-(1) A27 CDR1
V_LFR2	
WYQQKPGQAPRLLIF	
WYQQKPGQAPRLLIY	V_KIII 6-1-(1) A27 FR2
V_LCDR2	
GASSRAT	
GASSRAT	V_KIII 6-1-(1) A27 CDR2
V_LFR3	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	V_KIII 6-1-(1) A27 FR3
V_LCDR3	
QQYGRPGVT	
QQYGSSP	V_KIII 6-1-(1) A27 CDR3
V_LFR4	
FGQGTKVEIK	

RR2r3s4-1 scFv Amino Acid Sequence

Sequence (red letters indicate divergence from framework sequence)	**Variable Chain Name**
V_HFR1	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	
QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	V_H6-01 FR1
V_HCDR1	
SNIAAWH	
SNSAAWN	V_H6-01 CDR1
V_HFR2	
WIRLSPSRGLEWLG	
WIRQSPSRGLEWLG	V_H6-01 FR2
V_HCDR2	
RTYYRRSKWNYDYALSVKS	
RTYYRSKWYNDYAVSVKS	V_H6-01 CDR2
V_HFR3	
RINTNPDTSKNLFSLQLNSVTPEDTAVYYCTR	
RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	V_H6-01 FR3
V_HCDR3	
GGGRAHSA	
V_HFR4	
WGQGTLVTVSS	
$(Gly_4Ser)_3$ Linker	
GGGGSGGGGSRGGGS	
GGGGSGGGGSGGGGS	
V_LFR1	
EIVLTQSPGTLSVSPGERATLSC	
EIVLTQSPGTLSLSPGERATLSC	V_KIII 6-1-(1) A27 FR1
V_LCDR1	
RASQSVSRSHLA	
RASQSVSSSYLA	V_KIII 6-1-(1) A27 CDR1
V_LFR2	
WYQQKPGQAPRLLIF	
WYQQKPGQAPRLLIY	V_KIII 6-1-(1) A27 FR2
V_LCDR2	
GASSRAT	
GASSRAT	V_KIII 6-1-(1) A27 CDR2
V_LFR3	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	
GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	V_KIII 6-1-(1) A27 FR3
V_LCDR3	
QQYGRPGVT	
QQYGSSP	V_KIII 6-1-(1) A27 CDR3
V_LFR4	
FGQGTKVEIK	

Curriculum Vitae

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Education

1999-2004 **Massachusetts Institute of Technology** Cambridge, MA
Ph.D. Department of Chemical Engineering, February 2005. Thesis: Isolation and Engineering of a High Affinity Antibody Against P-selectin Glycoprotein Ligand-1 (PSGL-1).

1995-1999 **Princeton University** Princeton, NJ
B.S.E. Department of Chemical Engineering, Summa Cum Laude. GPA: 3.95/4.0. Certificate in Engineering Biology. Senior Thesis: The Role of Electrostatics in the Energetics of Interaction Between Proteins and Polymer Hydrogels.

Research Experience

MIT, Department of Chemical Engineering Cambridge, MA
Graduate Student (2000-2004)
Advisor: Professor K. Dane Wittrup

- Isolated and engineered a novel human single chain antibody (scFv) for use as a potential anti-inflammatory therapeutic using yeast surface display, fluorescence activated cell sorting (FACS), and directed evolution technology.
- Generated multiple high affinity antagonists of P-selectin adhesion.
- Engineered a large affinity improvement (at least 1 $*10^5$ fold)
- Developed a new method for the facile generation of large, shuffled antibody libraries in yeast.

Wyeth Research, Antibody Technologies Group and Cardiovascular and Metabolic Diseases Group Cambridge, MA
Summer Intern (2003 and 2004)
Supervisors: Gray D. Shaw and Angela Widom

- Generated IgGs based upon the scFvs engineered in the Wittrup Lab.
- Characterized the binding properties of the IgGs using ELISA and Biacore.

Princeton University, Department of Chemical Engineering Princeton, NJ
Summer Intern (1998)
Supervisor: Professor Jeffrey D. Carbeck

- Studied controlled release of proteins from polymer hydrogels.
- Gained extensive knowledge in the use of capillary electrophoresis as a tool to study modified proteins.

University of Pittsburgh Department of Chemical Engineering Pittsburgh, PA
Summer Intern (1997)
Supervisor: Professor Alan J. Russell

- Increased the stability of organophosphorus hydrolase, an enzyme that degrades nerve agents, in bleach.

Awards

2000-2004	NIH Biotechnology Training Grant Fellowship
1999-2000	Rosenblith Fellowship
1999	Phi Beta Kappa
	Richard K. Toner Thermodynamics Prize
	Sigma Xi Scientific Honor Society
1998	Tau Beta Pi Engineering Honor Society

Publications

Swers, J.S., Widom, A., and Wittrup, K.D. (2005) Characterization of a novel human anti-PSGL-1 antibody. In preparation.

Swers, J.S., Yeung, Y.A., and Wittrup, K.D. (2005) Integrated mimicry of B cell antibody mutagenesis by PCR and homologous recombination in yeast. In preparation.

Swers, J.S., Kellogg, B.A., and Wittrup, K.D. (2004) Shuffled antibody libraries created by *in vivo* homologous recombination and yeast surface display. *Nucleic Acids Res.*, **32**, e36.

Colby, D.W., Kellogg, B.A., Graff, C.P., Yeung, Y.A., **Swers,** J.S., and Wittrup, K.D. (2004) Engineering antibody affinity by yeast surface display. *Methods Enzymol.* **388**, 348-358.

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Bhatia, S.K., **Swers,** J.S., Camphausen, R.T., Wittrup, K.D., and Hammer, D.A. (2003) Rolling adhesion kinematics of yeast engineered to express selectins. *Biotechnol. Prog.* **19**, 1033-1037.

LeJeune, K.E., **Swers,** J.S., Hetro, A.D., Donahey, G.P., and Russell, A.J. (1999) Increasing the tolerance of organophosphorus hydrolase to bleach. *Biotechnol. Bioeng*. **64**, 250-254.