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Integrated Mimicry of B Cell Antibody Mutagenesis Using Yeast Homologous Recombination

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Abstract

Antibody affinity maturation proceeds in vivo via a combination of point mutations, insertions, deletions, and combinatorial shuffling of light chains or portions of the heavy chain, thereby reducing the probability of trapping in local affinity optima in sequence space. In vivo homologous recombination in yeast can be exploited to mimic the broad spectrum of mutational types deployed by B cells, incorporating both receptor revision and receptor editing together with polymerase-directed point mutagenesis. This method was used to effect a 10,000-fold affinity improvement in an anti-peptide single-chain antibody in three rounds of mutagenesis and screening, and a 1,000-fold affinity improvement in an anti-protein single-chain antibody in a single round. When recombinational mutagenesis (CDR or chain shuffling) was directly compared to error-prone PCR, the recombinational approach yielded greater affinity improvement with substantially reduced divergence from germline sequences, demonstrating an advantage of simultaneously testing a broad range of mutational strategies.

Keywords

Yeast display; Affinity maturation; Homologous recombination; Receptor editing; Receptor revision; Chain shuffling; Antibody engineering; Library design

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Introduction

B cells utilize a variety of mechanisms to generate antibody sequence diversity. Preimmune V(D)J recombination first creates CDR3 loops of varying length and composition [1]. Then, in response to antigen challenge, the antibody repertoire is further shaped by somatic hypermutation (SHM) [2], combinatorial replacement of entire light chains (receptor editing [3–5]), and exchange of portions of the V_H gene (receptor revision [6, 7]). Light chain exchange in the periphery has been observed in humans [4] and mice [5], and has been proposed to play a role in fine-tuning antigen binding affinity [8]. Receptor revision creates hybrid V_H genes that maintain the same complementarity determining region 3 (CDR3) while splicing in new coding sequence for framework 1 (FR1), CDR1, FR2, CDR2, and most or all of FR3 [9, 10]. Receptor revision and receptor editing cause larger movements in sequence space than SHM, potentially enabling the affinity maturation process to escape local energetic minima [11]. Amino acid insertions and deletions in CDRs occur frequently in vivo [12–14], and such changes are well tolerated by the variable region's framework architecture [15]. This 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 [16, 17].

By 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 [18–20], gene shuffling [18], targeted mutagenesis of CDR residues [21, 22], chain shuffling [23–25], and CDR shuffling [23, 24, 26]. Most in vitro antibody affinity maturation strategies employ error-prone PCR for mutagenesis, which can sample a varying frequency of nucleotide point mutations [19]. Random mutagenesis in conjunction with gene shuffling [18] and targeted mutagenesis of CDR residues [21] have yielded dramatic affinity improvements of three 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 [23] and less than 10-fold when directed against a protein antigen [24, 26, 27]. The mutagenesis methods just enumerated have been used previously in isolation, or in pairs. However, for a given antibody/antigen pair the particular categories of genetic diversity from which the greatest affinity improvements will be isolated cannot be identified a priori.

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 (chain shuffling). This approach has been applied to increase the affinity of an anti-peptide antibody by at least five orders of magnitude, from micromolar to picomolar 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. 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. We also compared recombinational mutagenesis (CDR and chain shuffling) to error-prone PCR for affinity

maturation of single-chain antibody fragments (scFv) with micromolar affinity against a protein antigen, and find that recombinational mutagenesis in this instance accomplished higher affinity improvements than those obtainable only through extremely heavy point mutagenesis (9–51 mutations per mutant scFv). Chain shuffling appears to be the most successful strategy with micromolar-affinity lead antibodies, while smaller structural alterations are more effective for fine-tuning binding sites from nanomolar to picomolar affinity.

Results

Integrated B Cell Mimicry Approach

To mimic B cell receptor revision [6, 7, 9, 10] and receptor editing [3–5, 8, 11], we devised a method to incorporate increasing segment lengths of the human V_H domain non-immune repertoire into a given scFv clone, using yeast homologous recombination [28] and the conservation of framework sequences to provide junctions between each of the CDRs (Fig. 1a, b, e; Table 1). PCR fragments of a single α-PSGL-1 human scFv clone were generated with 5′ ends within FR2 or FR3, and recombined with full-length scFvs constructed from a nonimmune human repertoire [29]. Diversity in such libraries is generated from nonimmune library cDNA, which codes for sequences already selected as being both compatible with the antibody fold and presumably functionally expressed in vivo. Although recombination at the termini of linear fragments is favored by yeast homologous recombination [28], sequence analysis indicates that crossover points internal to the transformed fragments added CDR loop diversity beyond the expected junctions in 30–40% of the clones (Table 1A, B). A fraction of the clones (2/10 CDR1 + CDR2 receptor revision library, Table 1B; 6/10 of the receptor editing library, Table 1C) contain only the V_L, with the V_H deleted 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. The presence of these V_L—only clones can enable serendipitous paratope mapping and consequent development of alternative domain antibody configurations [30].

Chain Shuffling for First Round Affinity Maturation of Micromolar-Affinity scFvs—Depending on the means of library construction and screening, it is not uncommon for either the V_H or the V_L domain alone of an scFv to predominantly contribute to antigen binding free energy [30, 31]. B cell repertoires from immunized animals or patients might be expected to possess significantly amplified subpopulations of B cell clones in which the heavy and light chains both contribute to binding affinity, and consequently a display library subcloned from this repertoire could sample productive chain pairings with higher probability. However in a non-immune scFv display library, the pairing of heavy and light chains originating from the same B cell clone would be a very rare event. In such instances, one would expect that substituting a diverse repertoire for the non-contributing variable domain could provide a rapid means for significant affinity improvement. Of course, the original pairing in a given B cell clone might not be recreated, but a more beneficial pairing is likely to exist than those found in the initial nonimmune library. We have found more beneficial pairings resulted in two instances in which recombinational chain shuffling was directly compared to error-prone PCR for affinity maturation of low affinity (μM K_d) scFvs.

In the first case, a previously described [29] yeast-displayed nonimmune antibody library was screened 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) [32] using fluorescence-activated cell sorting (FACS). After multiple sorts in the lead isolation round, round 0, sixteen unique clones were isolated and sequenced. K_d values for these clones were difficult to determine due to nonspecific ligand stickiness at high micromolar concentrations, however a lower bound apparent K_d estimate of 5 μM against the bivalent antigen was obtained for all of these clones (round 0 in Fig. 2). Previously, we have shown that K_ds determined by flow cytometry and yeast surface display are similar to those determined by Biacore [29]. The 16 clones were mutagenized together by error-prone PCR as described previously [33], and transformed together into yeast for a single multiplexing affinity maturation screen by flow cytometry. After an affinity maturation round of five sorts by FACS (round 1 in Fig. 2), multiple isolates of a single clone were isolated, and found to be a hybrid of the V_H from one of the original clones (with one CDR3 point mutation) and the V_L from a different clone (Table 2) indicating that receptor editing had likely occurred through in vivo homologous recombination within the $(gly_4ser)_3$ linker region shared by all of the scFv clones [28]. The predominance of this chain-shuffled clone is striking given the competition in the library from 4.8×10^6 mutated clones from 16 different lineages versus the limited potential diversity of 256 unique V_H/V_L chain pairings. It is possible that the best single clone was created by template switching during the PCR stage. However, regardless of how the clone was created, this example validates the concept of chain shuffling as a method of affinity maturation for low affinity binders. Evidence from our previous work [28] shows that the frequency of shuffling between proteins of similar sequence allows one to rapidly explore shuffled clones with relative ease through yeast homologous recombination.

Chain shuffling was also found to be the most successful strategy for affinity maturation of a micromolar-affinity lead human scFv against the human aspartyl (asparaginyl) β-hydroxylase (HAAH) ectodomain. The equilibrium binding constant of the initial clone was determined in the yeast-displayed format, and estimated to be >10 μM. Three different mutagenesis strategies were directly compared for affinity maturation of this scFv: error-prone PCR, CDR shuffling (Figs. 1a, b), and heavy chain shuffling (Fig. 1d). Seven higher affinity clones isolated from the error-prone PCR mutagenized library were sequenced and found to possess a strikingly high average of 25 ± 16 mutations, significantly higher than the number of mutations in the unscreened library (6.6 ± 8.0 for 17 unselected clones; difference statistically significant with $P < 0.001$). There were many clones that had more than 20 mutations but most of these clones had stop codons and were excluded from the statistical analysis. The dissociation constant of the best-improved clone was determined to be 120 ± 20 nM, a two order of magnitude improvement (Fig. 3). Upon closer examination of the amino acid sequences of these improved clones, cysteine deletion or insertion in the V_H domain was frequently observed. Five of the seven sequenced clones replaced the V_H22 cysteine residue with either arginine or tyrosine, abolishing the V_H intra-domain disulfide bond. Of these five clones, three have mutations introducing a novel cysteine in either the heavy chain FR3 or light chain CDR3. For the two clones that did not alter V_HCys22, one clone did not introduce a new cysteine, but had 19 amino acid changes. The other clone had a strikingly high 51 amino acid mutations, and also replaces a tyrosine with a cysteine in the

heavy chain CDR3 loop, just four residues away from the consensus V_H22 cysteine. This clone had point mutations distributed throughout, not sections of sequence that were completely different, indicating that this clone arose through random mutations and not template contamination during library construction. The frequent cysteine mutations and extraordinarily high number of mutations in the selected clones suggests that fairly dramatic structural change in the heavy chain architecture is required to improve the affinity of this clone's starting binding motif. The starting scFv has, respectively, zero and four mutations from germline heavy and light chain framework sequences. The clones isolated from the random mutagenesis studies have germline framework mutations ranging from 8 to 35, which would raise substantial concerns about immunogenicity in any potential therapeutic application of this antibody. In addition, the removal of the V_H intra-domain disulfide bond raises concerns about the stability of these clones.

A receptor revision library was constructed by replacing either heavy chain FR1 to CDR1, FR1 to CDR2, or FR1 to CDR3 of the WT α-HAAH scFv with the nonimmune heavy chain fragments (Fig. 1a–c, respectively) [28], with all three pooled into a single library for screening. After sorting, four unique affinity-improved clones were isolated, of which three had replaced FR1 to CDR3 with a sequence from the nonimmune library (effectively a full V_H chain shuffle event), and the remaining clone replaced the FR1 to CDR2 sequence. The dissociation constant of this CDR shuffled mutant (CM4) was 17 ± 5 nM, an over three order of magnitude improvement relative to the WT affinity, and tenfold better than the best clone selected from the random mutagenesis library. However, this CDR shuffled mutant possesses only two mutations from the germline heavy chain framework sequence, significantly fewer than in the random mutagenesis clones.

A heavy chain shuffled library was constructed by shuffling heavy chains from the nonimmune library against the WT light chain (Fig. 1d). Five clones from the unscreened library were sequenced and confirmed to have unique heavy chains (data not shown). Following six rounds of sorting, six clones were identified with unique heavy chains distinct from the initial scFv. The dissociation constants for two selected clones, LLm11 and LLm13, were determined to be 16 ± 4 and 26 ± 8 nM, respectively, a three order of magnitude improvement in affinity over the starting scFv (for example titrations, see Supplemental Fig. 2). The extent of the affinity improvement in this heavy chain shuffled library was tenfold better than in the random mutagenesis study, although the library sizes were 25-fold smaller than the random mutagenesis library. Similar to the CDR shuffled mutants, these clones also have minimal deviation from the germline framework sequence (on average 4 ± 2 mutations from heavy chain germline framework). Since it is usually the case that the heavy chain CDR3 determines specificity, competition assays were performed to verify that clones created by heavy chain shuffling bound to epitopes that overlapped with the parental clone (data not shown).

In both of these examples (α-PSGL-1 and α-HAAH), affinity maturation of a micromolar-affinity lead scFv was accomplished with the least divergence from germline sequences by using a chain shuffling mutagenesis strategy, in direct comparison with clones selected from error-prone PCR mutagenized libraries.

10^5-Fold Affinity Maturation in Three Rounds Using Integrated Mimicry Mutagenesis Strategy—Utilizing a combination of the strategies described here (chain shuffling, receptor revision, and error-prone PCR), an α-PSGL-1 scFv was engineered from a starting monovalent binding affinity > 10 μM to approximately 100 pM (Fig. 2, Table 2). The mutational class of the best clone in each round was: chain shuffling and point mutagenesis (round 1); point mutagenesis (round 2); and receptor revision with an insertion in CDR2 (round 3). For this antibody lineage, a different mutational strategy was most effective for each round of library construction and screening. The affinity improvements in each round can clearly be seen in Fig. 4 in which the bivariate histograms of the round by round affinity maturation are shown. It is important to note in Fig. 4 that divalent PSGL-1 was used for sorting in rounds 0–2 and monovalent PSGL-1 was used for sorting in round 3. Switching from divalent to monovalent antigen was necessary to achieve high affinity binding clones after round 2 because the antigen avidity makes affinity discrimination of binders difficult when the apparent affinity of the divalent antigen for a yeast-displayed scFv is in the picomolar range (Fig. 2). An example of a K_d determination by titration of monovalent PSGL-1 for the best clone from round 2 can be found in Supplemental Fig. 1.

It is noteworthy that chain shuffling/receptor editing, which was most successful in the first round, failed to create any isolatable improved clones in the final round, indicating that once the binding site had been optimized to some extent (50 nM K_d), complete substitution of half the molecule is too draconian of a strategy compared to more subtle replacements. It is important to note that random mutagenesis alone was able to improve a clone with micromolar starting affinity a thousand fold in round 2. This improvement might seem at odds with our previous assertion that chain shuffling, which only yielded a modest improvement in round 1, is more effective for the affinity improvement of lead antibodies than random mutagenesis in this case. However, it is essential to understand that in round 1 the antibodies were lead antibodies. Only once a beneficial chain pairing took place could affinity maturation proceed through random mutagenesis to yield large affinity improvements because a suboptimal light chain would require more drastic restructuring than an optimal light chain. Optimal chain pairings allow for rapid improvements by random mutagenesis.

The total monovalent affinity improvement after three rounds of mutagenesis is estimated to be over five orders of magnitude. For the best clone isolated in round 2, both the monovalent peptide-binding affinity, 50 ± 30 nM, and the divalent Fc fusion binding affinity, 90 ± 110 pM, were determined (Fig. 2). This comparison allows for calculation of an effective second site concentration of 28 μM in this system, according to avidity theory [34, 35]. The monovalent K_d for the lead scFvs isolated from the non-immune library can, therefore, be approximated as >10 μM (Fig. 2).

Discussion

Receptor revision and receptor editing both occur during affinity maturation in human germinal centers [3–11]. B cell immunoglobulin gene rearrangements in the periphery have been hypothesized to allow antibodies to make large leaps in sequence space in order to escape local optima on the affinity landscape [11]. These additional receptor diversification

processes are expected 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. As the sequence must pass through lower affinity intermediates which would be lost during affinity driven selection in order 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 receptor revision and receptor editing to swap in larger functional portions of the variable antibody domain, generating binding motifs with substantially altered structure without the detrimental consequences that might accrue from heavy point mutagenesis. In this way, B cells appear to be utilizing the “family shuffling” strategy, which has proven a successful strategy for in vitro directed evolution [36].

Affinity maturation by point mutagenesis is a validated method for in vitro affinity maturation. In contrast, large structural changes via amino acid insertion and deletion or swapping of whole genetic regions in a method mimicking receptor revision or receptor editing has not in general yielded significant results in the past. First, there is no facile method for constructing libraries with amino acid insertions and deletions because the sites for insertion or removal are not readily apparent a priori. Large scale replacement of whole regions of scFv DNA were successful in improving hapten binding by two orders of magnitude [23] but had lesser success against a protein antigen [24]. 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 slightly faster on rates [26]. Clearly, the success of any 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.

For the anti-HAAH scFv described here, affinity improvement by error-prone PCR mutagenesis required extremely high substitution rates, and the structurally important cysteine (H22) in the heavy chain was frequently mutated, implying that small structural changes were insufficient to improve the complementarity of interaction with the antigen. This high level of deviation from germline framework sequence in the isolated mutants could very well create immunogenic epitopes, and consequently in the absence of an alternative mutagenesis strategy this antibody lineage would be unlikely to be developed further. In contrast, recombinational mutagenesis generated mutants with substantial changes from the WT sequence while conserving a germline framework sequence in the mutants. Recombinational mutagenesis is superior in this case as the mutants thus generated have higher affinity improvement (three orders of magnitude) but far fewer framework mutations. In cases such as this, it is favorable for B cells to use recombinational mutagenesis over somatic hypermutation to generate improved mutants with substantial changes from the first generation antibody, while keeping the sequence similar to the germline framework sequence.

An integrated approach utilizing both random and recombinational mutagenesis was employed to affinity mature an anti-peptide scFv by over five orders of magnitude. This approach incorporated random mutagenesis to some extent in each round of affinity

maturation, due to the use of PCR, but also included receptor editing/chain shuffling in the first round and receptor revision plus amino acid insertion in the third round. Receptor editing was beneficial for low affinity clones. 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 two orders of magnitude affinity improvement [23]. When chain shuffling 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 [24]. As Tomlinson et al. demonstrated, diversity in the antibody primary repertoire is focused in the CDR3 of each chain and spreads outward to the periphery of the binding site during somatic hypermutation [17].

Although the exact strategy employed here may be pertinent only to these specific antibodies, the general concept that chain shuffling, random mutagenesis, amino acid insertions/deletions, and receptor revision are each in isolation and in combination useful strategies for affinity maturation and for the generation of panels of binders with different therapeutic potential is valid in general. The power of the yeast homologous recombination technique is that it allows for the exploration of all of these possibilities in a facile manner not possible through previous methods.

It is possible that methods such as chain shuffling and receptor revision, which lead to larger changes in the antibody binding site than random mutagenesis, are more likely to create antibodies that bind different epitopes on the target. Epitope switching could be detrimental in some cases where the binding region for therapeutic effectiveness is relatively small. However, it is also possible that an epitope switch could create an antibody of increased therapeutic value. As the yeast homologous recombination method allows for the easy exploration of many different strategies for affinity improvements, this method should increase the number of high affinity clones one has to choose from to evaluate for therapeutic efficacy.

We demonstrate here a facile method to greatly diversify the sampling of sequence space for affinity-improved antibodies. The strategy presented here is uniquely capable of straightforwardly mimicking in vitro the broad genetic spectrum generated by B cells during affinity maturation in vivo. The high fidelity method of in vivo homologous recombination in yeast [28] allows for the creation of hybrid genes between a known sequence and nonimmune library DNA without PCR assembly, ligation, *E. coli* transformation, or the introduction of recognition sequences for site-specific recombinases [25]. Our results suggest that incorporation of multiple mutagenic strategies lowers risk in affinity maturation, by sampling qualitatively different diversity at each stage. Chain shuffling/receptor editing was most effective in early rounds of affinity maturation but not when affinity has been improved to the nanomolar range. Recombinational mutagenesis can lead to antibodies with reduced framework divergence, different cross reactivity, and different therapeutic potential. Our integrated approach, made possible by the in vivo homologous recombination mechanism in yeast, should prove a valuable tool for antibody engineering.

Experimental Protocol

Materials

The non-immune library used to isolate anti-HAAH and anti-PSGL-1 binders has been previously described [29]. The HAAH protein and monoclonal antibody FB50, which recognize a linear epitope on HAAH, were provided from the Panacea Pharmaceuticals (Gaithersburg, MD). The divalent, biotinylated b.19.ek.Fc fusion protein and the human Fc were generous gifts of Dr. Raymond T. Camp-hausen, formerly of Wyeth (Cambridge, MA) [32]. The monovalent from of this antigen, b.19.ek, was a gift of Gray Shaw (Wyeth, Cambridge, MA). The yeast strain used for all experiments, EBY100, has been previously described [37]. The yeast surface display vector pCTCON, a derivative of the vector pCT302, has also been previously described [29]. Electro-competent *E. coli* Electro Ten-Blue® were purchased from Stratagene (Cedar Creek, TX).

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 chicken version of 9e10 was obtained from Molecular Probes (Eugene, OR). 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). The fluorophore goat anti-chicken Alexa488 was purchased from Molecular Probes (Eugene, OR).

Mutagenic PCR

Mutagenic PCR of both anti-HAAH scFv and anti-PSGL-1 scFv was performed similarly 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) [38, 39]. Since the plasmid backbones for both anti-HAAH scFv and anti-PSGL-1 scFv were the same, the same set of primers could be used for both PCR reactions. Primers were ordered from MWG Biotech (High Point, NC). The forward primer was 5′-CGACGATTGA AGGTAGATACCCATACGACGTTCCAGACTACGCTC TGCAG-3′ and reverse primer was 5′-CAGATCTCGAGC TATTACAAGTCTTCTTCAGAAATAAGCTTTTGTTC-3′. These primers have approximately a 50 base pair overlap with pCTCON that has been digested from *Nhe*1 to *Bam*H1. 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 min followed by either 5, 10, or 20 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, followed by 1 cycle of 72°C for 10 min. 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 μl was used in a 100 μl PCR

reaction with the following cycling conditions: 1 cycle of 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, followed by 1 cycle of 72°C for 10 min. 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.

CDR Domain Shuffled Library of Anti-HAAH scFv

The CDR domain shuffled libraries were prepared utilizing the homologous recombination in yeast [28]. First, fragments of non-immune human scFv library were amplified using the same set of HA and c-myc primers as the mutagenic PCR at 2.5 μM. 25 ng of human nonimmune library DNA (7.2×10^9 copies) and 0.5 μl of Taq polymerase (Invitrogen, CA) was used per PCR reaction. The reaction was annealed at 55°C for 1 min, extended at 72°C for 1 min, and cycled for 35 times. The 900 bp fragments from the PCR reaction containing the nonimmune human scFv flanked by HA and c-myc tags were then gel purified. Meanwhile, different fragments of anti-HAAH scFv #11 were prepared using primers targeting different heavy chain framework regions and sequence downstream of the c-myc tag. #11 fragment missing FR1 to CDR1 was generated using primer targeting heavy chain FR2 (5′-GGA TCAGGCAGTCCCCATCGAGAGGCCTTGAGTGGCTG GG-3′) and primer targeting sequence downstream of c-myc (5′-ACAGTGGGAACAA AGTCGATTTTGTTA CATCTACAC-3′). Similarly, fragments missing FR1 to CDR2 and FR1 to CDR3 were generated by replacing the previous FR2 primer with primer targeting FR3 (5′-CGA ATAACCATCAACCCAGACACATCCAAGAACCAGT TCTCCC-3′) and FR4 (5′-GG GGCCAGGGAACCCTGG TCACCGTCTCCTCAGGGA GTGCATCC-3′), respectively. 10 ng of WT scFv and 2.5 μM of primers were used in the reaction. The PCR reactions were performed using *Taq* polymerase at an annealing temperature of 54°C for 1 min, and at an extension temperature of 72°C for 38–65 s depending on the length of the desired fragments. The reactions were cycled for 35 times. The CDR domain shuffled libraries were then constructed by co-transforming the nonimmune human scFv fragments, fragments missing the desired shuffled portion (either FR1 to CDR1, FR1 to CDR2 or FR1 to CDR3) and a *Nhe*I-to-*Xho*I cut pCTCON backbone into EBY100 yeast.

Heavy Chain Shuffled Library of Anti-HAAH scFv

Nonimmune human heavy chain sequences were restriction digested out from the human scFv library plasmids using *Nhe*I and *Bam*HI, and then gel purified. Similarly, the WT anti-HAAH scFv plasmid was digested with *Nhe*I and *BamH*I to remove the WT heavy chain, and the plasmid fragments containing the WT light chain were gel purified. The nonimmune heavy chain fragments were then ligated into the cut WT backbone at a mass ratio of 7.5 to 1. The ligated products were then electroporated into the Electro Ten-Blue® competent cells according to the manufacturer's protocol. The plasmids were then recovered using miniprep kit and then electroporated into EBY100 cells.

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 *Bam*H1 cut site 5′ to the c-myc tag and a *Xho*1 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-immune library PCR products and to restrict 3′ homology to the cut vector to the r2s4-2 PCR products. The forward primer HA-for, 5′-GTTCCAGAC TACGCTCTGCAG-3′, which primes in the HA region of pCTCON and is well 5′ of the Nhe1 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′-CTTCTTCAGAAATAAGCTTTTG TC-3′, primes in the c-myc region and creates fragments that lack 3′ homology to an *Nhe*1 to *Xho*1 digested pCTCON backbone. For library RR1, r2s4-2 based PCR products were created using the forward primer FR2-for, 5′-GGATC AGGCAGTCCCCATCG-3′, which primes in framework 2 and for library RR2, r2s4-2 based PCR products were created using the forward primer FR3-for, 5′-CGAATAACCATCA ACGCAGAC-3′, which primes in framework 3. The reverse primer in both cases was past-*Xho*1-rev, 5′-GGGAAC AAAGTCGATTTTGTTAC-3′ which primers 3′ to the *Xho*1 site. The cutting of the backbone pCTCON from *Nhe*1 to *Xho*1 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 *Nhe*1 to *Bam*H1. 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 in every scFv. The non-immune library DNA was PCRed using the HA-for primer and a reverse primer c-myc-rev, 5′-CAGATCTCGAGCT ATTACAAGTCTTCTTCAG-3′. The non-immune library PCR products for the creation of a receptor editing library were digested from *Nhe*1 to *Bam*H1 to eliminate the 5′ homology to the cut pCTCON. There is a conserved *Bam*H1 site in most scFvs 5′ to the $(gly_4ser)_3$ linker thus cutting with *Bam*H1 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 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, followed by 1 cycle of 72°C for 10 min. All PCR fragments were purified using a Qiagen PCR purification kit.

Yeast Transformation

Insert fragments were concentrated with Pellet Paint (Novagen, Madison, WI) to a concentration of 5 μg/μl according to the manufacturer's instructions and cut pCTCON backbone was likewise concentrated to a concentration of 1 μg/ll. 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 electrocompetent yeast and their transformation has been described previously [40]. Transformation efficiencies were on the order of 10^5 transformants per microgram of insert DNA.

Yeast Growth and Induction

Detailed protocols for yeast growth and induction can be found in Boder and Wittrup [40].

Flow Cytometry for Sorting and K_d etermination on the Yeast Surface

All sorting was performed on a MoFlo cell sorter (Cytomation, Fort Collins, CO).

The library size of the anti-HAAH scFv random muta-genesis library was 3×10^6 and this library was sorted six times. The anti-HAAH CDR domain shuffled libraries were of size 1.1×10^4, 1.2×10^4, and 1.1×10^4, and were pooled together for screening. Six rounds of screening were performed using FACS stepping down from 500 to 160 nM of HAAH antigen to screen for improved binders and without antigen to eliminate any binder against the detection antibodies. The anti-HAAH heavy chain shuffled library was of size 1.2×10^5. Six rounds of sorting, either stepping down from 800–500 nM of HAAH to screen for improved binders or without any HAAH to eliminate any binder against the detection antibodies, were performed using FACS.

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 [29] 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, tenfold excess human Fc was added as a competitor in the second and third sorts to prevent isolation of Fc binders. 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 DNA of all 16 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 [41] 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 KPL-1 (an anti-PSGL-1 monoclonal antibody that inhibits binding of P-selectin to PSGL-1, BD Biosciences, San Jose, CA) 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 article). For the anti-HAAH scFv, cells were labeled first with HAAH at the appropriate concentration (titrations were performed at 8-10 concentrations covering approximately 3–4 logs with the midpoint concentration at approximately the concentration of the K_d) for 1 h at 37°C. Cells were then washed with cold PBS, and incubated with chicken 9e10 at a dilution of 1:100 and 300 nM of FB50 for 20 min at 4°C. After another washing with cold PBS, cells were incubated with goat anti-chicken Alexa 488 and goat anti-mouse PE at dilutions of 1:66 and 1:50, respectively, for 15 min at 4°C. These cells were analyzed after a wash with PBS. For the anti-PSGL1 scFv, cells were labeled with either b.19.ek.Fc or b.19.ek at the appropriate concentration (titrations were performed at 8–10 concentrations covering approximately 3–4 logs with the midpoint concentration at approximately the concentration of the K_d) for ½ h at 37°C. After a wash with 1 ml of cold PBS, the yeast were incubated ½ h on ice with 9e10 in a 1:100 dilution in PBS. After a cold PBS wash, the yeast were incubated ½ h 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 [40]. The method for performing a titration to determine the K_d of yeast surface displayed scFvs has also been described previously [40]. Specificity for the N-terminal 19 amino acids of PSGL-1 was confirmed by observing the absence of biotinylated divalent PSGL-1 binding to the best clone from round 1 displayed on the yeast surface in the presence of a tenfold excess of the anti-PSGL-1 antibody KPL-1. Control yeast that did not express scFv did not show binding to divalent PSGL-1 or HAAH at the concentrations used in the K_d measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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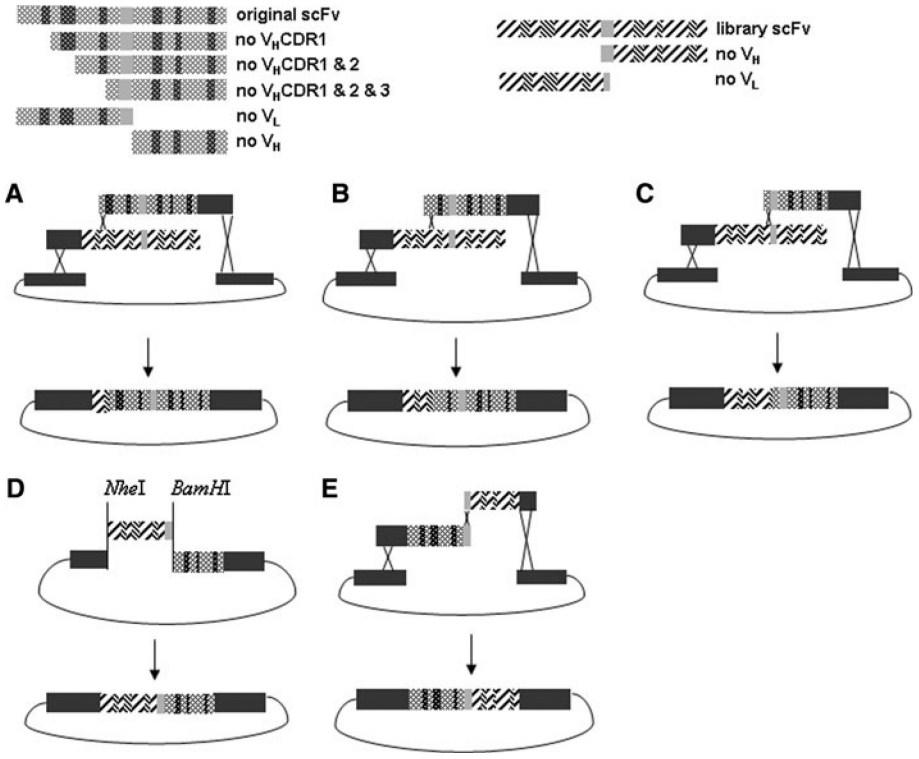

Fig. 1.

Schematic representation of mutagenesis strategies. The order of the regions in the scFv from left to right are V_HFR1, V_HCDR1, V_HFR2, V_HCDR2, V_HFR3, V_HCDR3, V_HFR4, $(gly_4ser)_3$ linker, V_LFR1, V_LCDR1, V_LFR2, V_LCDR2, V_LFR3, V_LCDR3, and V_LFR4. All libraries except **(d)** employed in vivo homologous recombination in yeast, while ligation was used in **(d)**. **a** V_HFR1 + V_HCDR1 are preferentially exchanged. **b** V_HFR1, V_HCDR1, V_HFR2, V_HCDR2 are preferentially exchanged. **c** V_HFR1 to V_HCDR3 are preferentially exchanged. **d** Heavy chain shuffling. **e** Light chain shuffling. **(a)** to **(d)** were the mutagenesis strategies employed in the affinity maturation of the anti-protein scFv. Meanwhile, receptor revision libraries (**a** and **b**) and the receptor editing library **(e)** were used to affinity mature the anti-PSGL-1 scFv

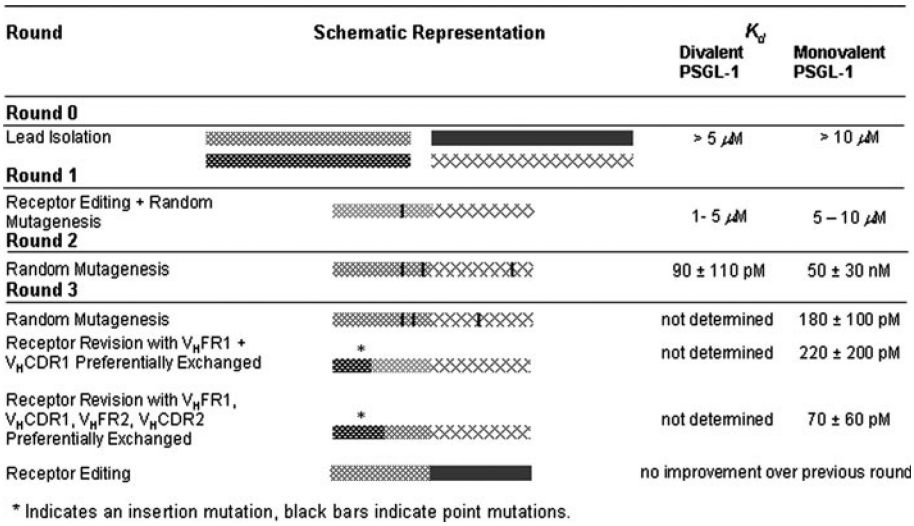

Fig. 2.

Round by round schematic of affinity maturation methodology with best clone K_d values for anti-PSGL-1 antibodies. A pool of scFvs was isolated in round 0 (named round 0 because it was a discovery round, and subsequent rounds are numbered 1, 2, 3 to represent the first, second, and third affinity maturation rounds, respectively) and affinity matured over 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. The and bars in round 0 represent the clones that contribute the heavy and light chains for subsequent rounds. The bar in round 3 indicates new DNA contributed through the receptor revision process. The new light chain in the round 3 receptor editing clone is shown in solid black

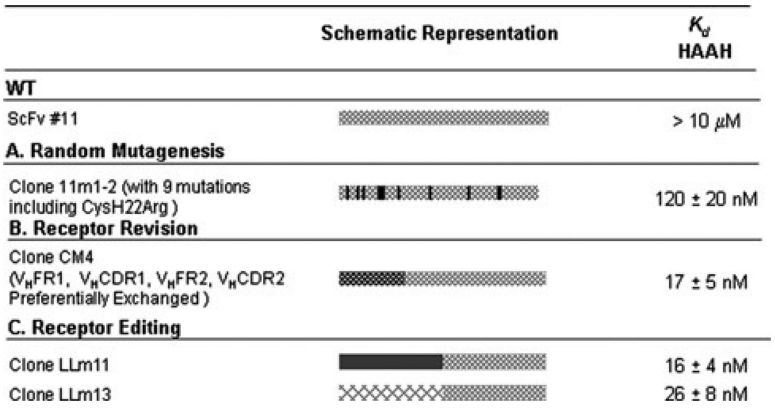

Fig. 3.

Topology and affinities of WT anti-HAAH scFv and mutants isolated from different mutagenesis strategies. Recombinational strategies (receptor revision and receptor editing) produced tenfold better affinity improvements with significantly fewer germline framework substitutions

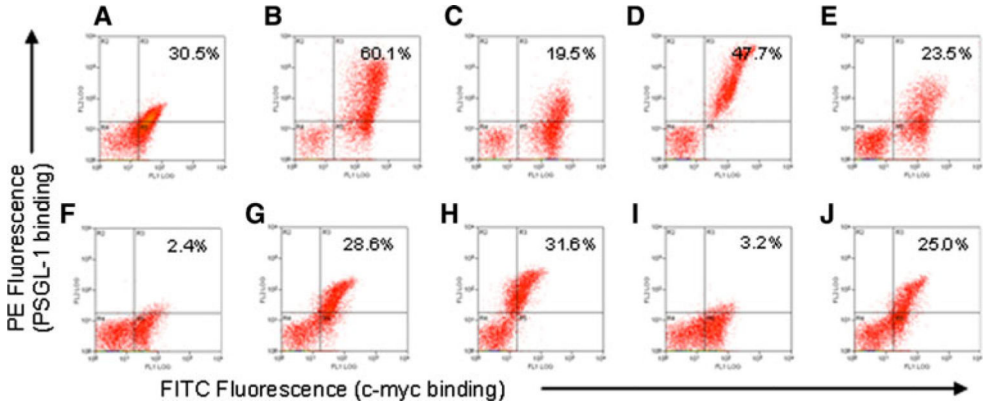

Fig. 4.

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. Binding to the biotinylated PSGL-1 antigen is detected by streptavidin-PE. The commercially available antibody 9e10 binds to the c-myc epitope tag located between the scFv and its attachment to the yeast cell. Goat anti-mouse FITC detection of bound 9e10 allows for normalization of the binding signal based upon expression level. **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 V_HFR1 + V_HCDR1 preferentially exchanged library, 0.5 nM monovalent PSGL-1. **h** Best clone from round 3 V_HFR1, V_HCDR1, V_HFR2, V_HCDR2 preferentially exchanged library, 0.5 nM monova-lent PSGL-1. **i** Best clone from round 3 light chain shuffled library, 0.5 nM monovlanet PSGL-1. **j** Best clone from round 3 mutagenic library, 0.5 nM monovlanet PSGL-1

Table 1

CDR amino acid sequences from unselected receptor revision and receptor editing libraries

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	SN**SA**AWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	SN**SAT**WN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	**G**N**SAT**WN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	**T**NN**T**AWN	RTYYRSKWYND**S**A**V**S**M**KS	**D**GG**LGPQR**
1	SN**AAG**WN	RTYYRSKW**ST**DYA**V**SVK**G**	**ARWGGLERRLYHFDF**
1	**RFAVS**	**VIPMFGTPKYAQRFQG**	**DAARGYGSGIEAFDI**

B. Round 3: receptor revision with V_HFR1, V_HCDR1, V_HFR2, V_HCDR2 preferentially exchanged[a]			
# of clones	**V_HCDR1**	**V_HCDR2**	**V_HCDR3**
Original	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRAHSA
1	SN**TA**AWN	RT**C**YRSKWY**F**DYA**P**SVKS	GGGRAHSA
1	SN**TA**AWN	RTYYRSKWYNDYA**V**SVKS	GGGRAHSA
1	**HDGA**AWN	RTYYRSKWY**YG**YA**I**SVKS	GGGRAHSA
1	SN**TA**AWN	RTYYRSKWY**S**DYA**V**S**L**KS	GGGRAHSA
1	**NTDA**AWH	RT**NF**RSKW**S**NDYA**LF**VKS	**QYGWNLGH**
1	SN**SA**AWN	RT**H**YR**F**KWY**S**DYA**V**SVKS	**GSKSSFDY**
1	SN**SAI**WN	RTYYRS**Q**WYNDYA**V**SVKS	**DLAGFDY**
1	**DYAMH**	**GISWNSGSIAYADSVKG**	**DIRGYYDSSGGFDP**

C. Round 3: receptor editing[b]				
# of clones	**V_LCDR1**	**V_LCDR2**	**V_LCDR3**	**Chain name**
Original	RASQSVSRSHLA	GASSRAT	QQYGRPGVT	A27
1	RASQSVS**SS**Y**L**A	**D**ASN**R**AT	QQ**RSDWPR**T	L6
1	RASQSVS**SS**Y**L**A	**S**ASSRAT	QQ**H**G**SSPY**T	A27
1	**TGTSSDVGGYNCVS**	**DVTKRPS**	Q**S**Y**D**G**SNHAV**	V1-3
1	**RTSQSIYRYLN**	**AV**SS**LQ**T	QQ**LKSYPRIT**	O2

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

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

Table 2

Amino acid sequences of the CDRs of the best clones isolated in each round of affinity maturation against PSGL-1

Clone Name	V_HCDR1	V_HCDR2	V_HCDR3	V_LCDR1	V_LCDR2	V_LCDR3	Other Mutations
A. Round 0							
r0s3-6	SNNVAWN	RTYYRSKWYNDYAASVKS	EGGRAQSA	RASQRVSTTYLA	AASRRAT	QHYRSSPPLT	
r0s3-15	*SYYWT*	*YIHNSGSTNYNPSLKS*	*RLKSGWFAGWFGP*	*RASQSVSRSHLA*	*GASSRAT*	*QQYGSPGVT*	
B. Round 1							
r1s6-15	SNNVAWN	RTYYRSKWYNDYAASVKS	**G**GGRAQSA	*RASQSVSRSHLA*	*GASSRAT*	*QQYGSPGVT*	
C. Round 2							
r2s4-2	SNNVAWN	RTYYRSKWYNDYAASVKS	GGGRA**H**SA	*RASQSVSRSHLA*	*GASSRAT*	*QQYG**R**PGVT*	Linker G to R
D. Round 3							
r3s4-3	SNNVAWN	RTYYRSKWYNDYAASVK**G**	GG**S**RAHSA	*RASQSVSRSHLA*	*G**V**SSRAT*	*QQYGRPGVT*	
RR1r3s4-2	SN**TA**AWN	RTYYR**R**SKWYNDYAASVKS	GGGRAHSA	*RASQSVSRSHLA*	*GASSRAT*	*QQYGRPGVT*	VHFR1 Qto H
							VHFR1 V to I
RR2r3s4-1	SN**IA**AW**H**	RTYYR**R**SKW**NY**DYA**L**SVKS	GGGRAHSA	*RASQSVSRSHLA*	*GASSRAT*	*QQYGRPGVT*	VHFR3 T to N
							VHFR3 A to P
							VHFR3 Q to L
							VHFR3 H to Q