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Anti-idiotypic Monobodies for Immune Response Profiling

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Abstract

A major goal in the study of autoimmune disease is the identification of biomarkers of disease to allow early diagnosis and initiation of treatment. The production of autoantibodies is the key feature of most autoimmune disease, so much effort has focused on characterizing the antigens reactive with these antibodies. However, even for the most well understood autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus, identification of antigens that detect autoantibodies in all patients have yet to be discovered. We describe a novel strategy for deriving mimotopes to disease-specific serum antibodies by selecting anti-idiotypic monobodies from a large molecular diversity library. Monobodies are derived by partial randomization of two surface exposed loops of a fibronectin domain scaffold in a phage display vector. The phage library is selected for binding to serum antibodies using a subtractive panning strategy. We evaluated this strategy by selecting the monobody library on a pool of serum immunoglobulin derived from a group of rheumatoid arthritis patients and evaluated selected clones for multi-patient reactivity and specificity for rheumatoid arthritis. The use of the fibronectin scaffold to derive stable, easy to produce molecular probes for diagnosis of autoimmune disease could be of significant value in improving diagnostic assays for virtually any disease that exhibits a characteristic immune response.

Keywords

Autoantibodies; Phage Display; Scaffold Protein; Mimotopes

1. Introduction

The diagnosis, understanding and evaluation of the course of most if not all systemic autoimmune diseases is hampered by the lack of sensitivity and/or specificity of autoantibodies. Disease-specific autoantibodies such as anti-dsDNA and anti-Sm (for systemic lupus erythematosus, SLE), anti-cyclic citrullinated peptide (CCP, for rheumatoid arthritis, RA) have a sensitivity that ranges from <25%–70% (for anti-Sm in SLE and anti-

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CCP in RA, respectively [1,2,3]. Even in entities like the anti-phospholipid antibody syndrome, defined and directly mediated by autoantibodies, the corresponding autoantibodies can only be detected in about 60% of patients at any given time and no autoantibody system has predictive ability or strong correlation regarding disease activity [4]. Moreover, no specific autoantibody systems have been identified for a number of other systemic diseases such as Sjögren's syndrome or psoriatic arthritis [5].

Several groups have demonstrated that molecular probes against specific immune responses can be generated by repertoire selection of gene fragment display libraries on patient sera [6,7,8]. With sufficiently large collections of probes derived from such selections, it is possible to obtain reliable diagnostic assays even for diseases not known to generate an immune response. This approach has also been used successfully to define antibody profiles in autoimmune disease. Most studies have used phage T7 display of cDNA derived from disease tissue as the source of probes to identify putative disease-associated antibody responses. However, it has been observed that up to 90% of the probes identified by these approaches are actually not derived from protein coding cDNA, but rather from 3' untranslated or frameshifted sequences, so they presumably represent mimotopes of the actual antigen [6,9]

There are a number of significant disadvantages to using cDNA-derived protein fragments as probes due to challenges posed by correct display of the peptides. These clones may exhibit multiple conformations resulting in lower affinity towards the recognizing antibodies. As a consequence, when building an assay based on such probes, the specificity and sensitivity of serum reactivity may be compromised. Moreover, each probe represents a unique protein fragment, greatly increasing the eventual cost and complexity of assay manufacture and quality control. Finally, a number of antigenic structures will simply not be reproduced by expression of gene fragments on the phage surface, including modified peptides [10] cell surface proteins, glycopeptides and polysaccharides.

One potential solution to the display of cDNA-derived proteins is the use of peptide display libraries. While easy to generate and enrich, peptides in general are not convenient for implementation into diagnostic assays as selected sequences must be synthesized and then coupled to suitable carrier or reagent proteins. Furthermore, the affinity of selected peptides is frequently quite modest due to the relatively small interface surface with the antibody, as well as being demonstrated to not adequately mimic antigen structure for antibodies that recognize discontinuous epitope [11].

The alternative approach we have developed eliminates these disadvantages by avoiding cDNAs entirely as a source of antigens and generating antigen surrogates derived from a single, stable protein scaffold. The tenth type III domain (FN) of human fibronectin has been developed by Koide and colleagues [12,13] as an alternative to antibodies to generate binding partners to proteins using site directed mutagenesis of two short surface exposed loops of the protein and phage or yeast display selection for binders. Applying standard phage display methodology [14] as modified in our laboratory [15,16], we have extended the utility of the FN scaffold libraries to demonstrate highly efficient enrichment on a diverse panel of antibodies, including several broadly neutralizing anti-HIV monoclonal antibodies (Mabs; b12 [17], PG9 and 16 [18], 4E10, F105 and Z13 [19]) to derive anti-idiotypic monobodies (α IMs) specific for these antibodies (Sullivan et al., manuscript in preparation). As with anti-idiotypic antibodies, some of the α IMs are predicted to be mimotopes of the original antigen. These α IMs should function analogously to the cDNA derived probes described above. However they have distinct advantages when formatting these probes into an assay due to the unusual stability of the fibronectin domain [12] and its efficient and inexpensive synthesis as a recombinant protein in *E. coli*.

We have chosen to evaluate the feasibility of this approach using rheumatoid arthritis as a model system. Rheumatoid arthritis is the most common inflammatory joint disease and the subject of intensive analysis of autoantibodies present in patients. Autoantibodies have been studied in RA to identify the antigenic targets and to discover RA-associated autoantibodies that can be used as disease markers. The discovery of antibodies directed against citrullinated peptides has led to several iterations of diagnostic assays for these antibodies, including tests for cyclic citrullinated peptides (CCP) [20,21,22] and mutated citrullinated vimentin (MCV) [23] that are commercially available. However, as many as one-third of RA patients test negative for these autoantibodies, highlighting the need for more accurate diagnostic assays. Moreover, with an increasing number of immune modulating therapies used for treatment of RA, there is an urgent need for new methods to determine which patients might benefit from particular therapeutic regimes as well as tools to monitor therapeutic efficacy.

Here we demonstrate that using a small, stable scaffold to display diverse amino acid sequences within two surface exposed loops of the protein, standard phage selection technology can be used to enrich for variants that react antibodies present in autoimmune patient serum. Several clones react with multiple patient antibodies, suggesting that the selected variants are mimicking antigens important in the disease process.

2. Methods

2.1 Generation of the Monobody Library

The FN scaffold was cloned into pAP-III6FL, a derivative of pAP-III6 [15] containing a fulllength gene III of M13, downstream of a FLAG epitope sequence. This plasmid was introduced into the CJ236 (*dut ung thi-1 relA1 spoT1 mcrA/pCJ105 /F' cam^r*) strain and single-stranded DNA (ssDNA) containing uracil was produced by infection with VCS M13 helper phage (Agilent). ssDNA from the phagemid particles was purified using a QiaPrep Spin M13 kit (Qiagen). A stop-template derivative containing Ase I restriction sites and in-frame stop codons (ATTAAT) within the BC and FG loops was generated by site-directed mutagenesis [24] and confirmed by sequence analysis. For library construction, the stop template was annealed to mutagenic oligonucleotides targeting the BC and FG loop in a large-scale reaction as described [25]. The library oligonucleotides (BC: 5'-CGTGATACGGTAATAACGMDNMDNMDNMDNMDNMDNMDNMDNCCAGCTGATCAGCAGGCT and FG: 5'-AATCGAGATTGGCTTGGAMDNDNMDNMDNMDNMDNMDNMDNAGTAACAGCATATAC AGTGATGGT) partially randomized seven positions in the BC loop and eight positions in the FG loop using NHK codons. Ten micrograms of template was annealed with phosphorylated library oligonucleotides, and after extension, the purified mixture was electroporated into TG1 cells (Agilent) and $\sim 5 \times 10^8$ ampicillin resistant transformants were obtained. Colony PCR followed by restriction digestion of the FN insert with Ase I revealed that about 40% of the colonies obtained had incorporated both oligonucleotides, yielding a functional library size of about 2×10^8 . The colonies were re-suspended by scraping the plates with LB broth and an aliquot containing >10 times the number of transformants was sub-cultured and grown in LB medium at 37° C for two hours. Ten mls of this culture were infected with M13 hyperphage [26] (Fitzgerald Industries) to generate multivalently displayed FN variants. The infected culture was diluted into 200 ml of LB containing ampicillin (100 µg/ml) and kanamycin (70 µg/ml) and grown overnight at 30 °C. Phage from the culture supernatant was harvested by precipitation with polyethylene glycol, re-suspended in 50 mM Tris-HCl, pH7.5, 150 mM NaCl (TBS) containing 0.5% casein and 15% glycerol, and frozen in aliquots at -80 °C.

2.2 Patient samples

Serum was collected at the University of Rochester Medical Center from 24 patients diagnosed with rheumatoid arthritis. Serum was collected, aliquoted and stored at -80°C . All patients provided signed written informed consent. All procedures and methods were approved by the Research Subjects Review Board at the University of Rochester Medical Center. None of the 24 rheumatoid arthritis patients included in this study were being treated with biological therapy, such as anti-TNF or Rituximab. Only 7 of the patients were receiving oral corticosteroids, and only 3 were being treated with methotrexate at time of sampling. Half of the patients had been diagnosed with RA for only 4 or fewer years.

2.3 Library Selection against serum IgG

The initial round of library enrichment was performed with Serum IgG captured in microtiter wells coated with goat anti-human IgG (H+L) (Thermo). The antibody was immobilized in 4 microtiter dish wells at $50\text{ }\mu\text{g/mL}$ in 50 mM TrisHCl , pH8, 150 mM NaCl (TBS) overnight and blocked with TBS containing 0.5% casein for one hour. Serum (either pooled normal serum (Innovative Research) or patient samples) was diluted in TBS + casein ($1\text{ }\mu\text{L}/50\text{ }\mu\text{L}$) and incubated for an additional hour at room temperature with shaking. After washing with TBS, the phage library was first added to the wells with normal serum for 1 hour, then transferred to the patient IgG wells and incubated with shaking for 2 hours. Phage were then removed and the wells were washed with TBS+0.5% Tween 20 seven times over 20 minutes followed by one wash with water. Bound phage were eluted with 0.1 M glycine HCl , pH 2 + 0.1% bovine serum albumin for 15 minutes. The eluate was removed, neutralized with Tris base, transduced into mid-log TG1 cells and plated on LB plates containing ampicillin.

The next day, colonies were scraped from plates in 5 mL of LB and sub-cultured into fresh medium for production of the next round of phage. One mL aliquots of mid-log culture were infected with hyperphage helper and grown as described above. Second and third rounds of phage were prepared from 30 mL cultures as described above and $50\text{ }\mu\text{L}$ aliquots were applied to single wells containing immobilized patient IgG. For the second round, biotinylated protein G (Thermo) was used to capture serum IgG after prior immobilization in pre-coated streptavidin wells (Pierce). The biotinylated protein G (0.5 mg/mL) was diluted 1:100 into TBS+casein and allowed to bind to the streptavidin-coated wells for 30 minutes. After rinsing with TBS, diluted serum was applied to the wells for 1 hour, washed and $50\text{ }\mu\text{L}$ of phage applied to the well containing pooled normal serum. After 1 hour, the phage were transferred to the well with patient serum and incubated for 2 hours. The well was washed as described above and the eluted phage were titered by transduction and a third round pool was prepared. For the third round, the same goat anti-human IgG (H+L) (Millipore) was used to capture serum IgG as described above.

2.3 Phage ELISA and Sequence Analysis

Individual clones after the second or third round of enrichment were tested by phage ELISA to confirm binding to patient serum IgG and lack of reactivity to IgG from pooled normal serum using goat-anti-human IgG to capture normal and patient serum as described above for the first round of enrichment. Phage from individual clones were prepared by PEG precipitation from 1.2 mL cultures, re-suspended in 0.3 mL in TBS+0.5% casein and $50\text{ }\mu\text{L}$ was added to the wells. After 1 hour incubation at room temperature, the wells were washed 10 times and a 1:2000 dilution of anti-M13-Horseradish peroxidase (HRP) conjugate was added for 1 hour incubation. After washing, HRP substrate (3,3',5,5'-tetramethylbenzidine, TMB, Kirkegaard and Perry Laboratories) was added and color was allowed to develop and the plate was photographed. Optionally, the reactions were terminated by acidification and the absorbance was measured on a plate reader. The αIM inserts of positive clones were

PCR amplified using flanking vector primers and sequenced at the University of Rochester Functional Genetics Core facility using a ABI BigDye® Terminator v3.1 Cycle Sequencing Kit. Excess dye terminators are removed with the Agencourt CleanSEQ system and reactions are run on the ABI 3730 DNA Analyzer with analysis performed using ABI Sequence Analysis Software and the KB Basecaller.

2.4 Soluble α IM expression and purification

Selected α IM variants were sub-cloned into a pET22 vector (Novagen) by introducing a methionine codon in front of the FLAG epitope as part of an NdeI restriction enzyme site. The carboxy terminus of the α IM was modified to add a *birA* site and the modified α IM was cloned between the NdeI and XhoI sites of the vector, adding a His6 purification tag immediately following the *birA* site (Sullivan et al., in preparation). Cultures were grown in LB to mid-exponential phase and protein expression was induced for 2 hours by addition of 1 mM IPTG and 50 μ M biotin. Cells were harvested and lysed with Bugbuster® reagent (Novagen) and purified by nickel affinity chromatography using Ni²⁺ magnetic beads and a Thermo Kingfisher instrument. Proteins were eluted from the beads with TBS containing 250mM imidazole and stored at 4°C. The purity of the proteins was determined by SDS-PAGE and Simply Blue staining (Invitrogen).

2.5 Immunoreactivity analysis with purified α IMs

Purified, biotinylated α IMs were diluted into TBS containing 0.5% casein and applied to streptavidin-coated immunoassay strips (Pierce) at a saturating concentration as determined by anti-Flag reactivity. After a 30 minute immobilization, the strips were rinsed and dilutions of subject or pooled normal sera in TBS plus casein were applied to the wells in duplicate. After an hour incubation with shaking, the strips were rinsed as described above for phage ELISA and the wells were incubated with anti-human IgG –HRP conjugate (KPL Laboratories). After an additional 60 minute incubation, the strips were washed and developed as described above. After acidification, the strips were read in a plate reader and the absorbance at 420 nm was plotted against dilution.

3. Results

3.1 α IMs selected on monoclonal antibodies can mimic antigen contact interactions

We have been exploring the use of fibronectin (FN) domain scaffolds containing two partially randomized loops to obtain antigen surrogates for characterizing antibody repertoires. We have validated this concept using a variety of monoclonal antibodies as targets (Sullivan et al., manuscript in preparation). Some of the surrogates selected from libraries constructed with the BC and FG loops of the FN scaffold (Figure 1) are expected to be actual mimotopes of the antigen. As shown in Figure 2, one such α IM selected on the broadly neutralizing b12 antibody directed against the HIV envelope accurately mimics the envelope with respect to the interaction with three key residues of the antibody known to be important for envelope binding [27], competes with gp120 for binding to b12 with a K_d of approximately 30 nM. This is in contrast to the well-studied peptide mimotope B2.1 [28], which despite high affinity and specificity towards the antibody, fails to interact with these same amino acids of the antibody paratope [29]. This success with selecting α IMs on individual antibodies led us to consider performing similar selections on serum IgG as an alternative to the use of cDNA display to derive probes of disease-specific immune responses in autoimmune disorders.

3.2 Selection of the Monobody Library on RA-positive Serum IgG

We utilized a subtractive panning scheme coupled with capture of serum IgG onto antibody or protein G immobilized in microtiter wells to efficiently select α IMs specific for an individual subject's IgG. Prior incubation at each round of enrichment on capture wells loaded with pooled normal serum IgG suppressed the isolation of both clones reactive with normal serum IgG or to the capture element itself provided that we changed the capture elements for each round. Thus, we used the goat antihuman IgG for the initial round, then switched to protein G for the second and finally repeated the use of the anti-human IgG monoclonal antibody for the third round, relying on a presumably uniform coating of the capture element and excess serum IgG to immobilize constant amounts of the target IgG repertoire. This process resulted in evident enrichment as detected by the increase in the number of eluted phage after three rounds, which had increased 10–100 fold relative to the first round. While this enrichment is substantially less than the 1000–5000 fold increase observed using single monoclonal antibody targets (Sullivan et. al, manuscript in preparation), it seems reasonable considering the diverse number of antibodies present in the well. Analysis of individual clones as shown in Figure 3, led to identification of a variety of α IMs reactive with the selecting patient serum, but we found that none that cross-reacted with any sample other than the one used in the selection (data not shown). This is not unexpected as we anticipated most antibodies unique to an individual would not be disease-associated.

3.3 Selection of the Monobody Library on pooled RA-positive Serum IgG

To focus the enrichment on disease-associated antibodies, we pooled serum from 24 RA-positive individuals and repeated the library enrichment. As with selection on single subject IgG, we observed evident enrichment after the second round and third round as revealed by phage ELISA (Figure 4). We screened 80 third round clones by phage ELISA and found ~80% were positive for reactivity with pooled RA serum IgG and negative for binding to pooled normal IgG. These were then sequenced to eliminate identical clones. We found approximately 40% of the ELISA-positive α IMs were identical, and the remaining clones consisted of 25 unique sequence clones, one clone found twice and two clones that were present in triplicate.

We next tested the unique sequence clones for reactivity to each serum sample in the pool to identify clones reactive with more than one subject. The majority of the clones reacted strongly with a single sample (data not shown), but a subset of 5 exhibited strong reactivity with two or more samples and weaker reactivity with additional samples. The results for a representative set of these clones are shown in Figure 5 along with a largely mono-reactive clone (3–13). Two additional multi-reactive clones had a pattern similar to 3–40 and 3–65, despite dissimilar sequences in the loops of the α IM (2–26: BC-SPPSPPS, FG-FASFP HDL and 3–71: BC-FFPEIEV, FG-FLHLP HQI). Searching the human proteome with the sequences of the BC and FG loops of the multi-reactive clones revealed frequent matches with four to six residues of the α IMs, but no obvious homologies to proteins known to be associated with autoimmune disease were evident.

While the use of the phage-displayed form of the α IMs is most convenient and inexpensive, implementation of this technology is likely to utilize expressed α IMs immobilized on a solid phase. We demonstrate that this format is also feasible as shown in Figure 6. We expressed clone 3–40 α IM containing a single biotin near the C-terminus along with a negative control α IM and immobilized both on streptavidin coated microtiter strips. We then titrated serum from one of the subjects that strongly reacted with the 3–40 α IM on phage and control pooled normal serum. The results indicated that in this format, strong reactivity specific for the 3–40 α IM is readily detected and the α IM is non-reactive with normal serum IgG.

4. Discussion

The development of probes to detect disease-associated immune responses by selection of display libraries on serum IgG has great potential to identify important reagents for a large class of diseases lacking good diagnostic assays. In addition to autoimmune diseases where an autoreactive antibody response is expected, there are indications that specific antibodies might be present in a variety of cancers [30,31] and other illnesses such as pre-eclampsia [32], heart disease [33] and type I diabetes [34]. While much of the work in this field has focused on the use of cDNA or peptide display to derive such probes, many results suggest that most of the selected probes are not derived from actual protein coding regions and are thought to represent mimotopes of the actual antigens. We were therefore interested in determining if display of an artificial protein could serve as an effective source of probes to characterize the autoantibody repertoire. A major challenge with any of these display technologies is to convert the informative probes from the phage setting into a useful reagent that can be robustly manufactured and incorporated into a commercial product. The key requirement for success in any of the antibody repertoire selection approaches is to identify probes that recognize a sufficiently large fraction of patients such that, by assembling a number of such probes that individually may only recognize only a subset of patient antibodies, can provide high specificity and sensitivity when sufficient numbers of probes are employed. However, this of necessity will increase the challenges in commercialization since each probe will represent a distinct chemical entity. We reasoned that by starting from a highly stable, economical to produce scaffold would facilitate translation into a commercial product with reduced costs associated with manufacturing and quality control. Moreover, the ability to derive probes for antibodies to non-native proteins, such as modified peptides or altered cell surface features might result in new biomarkers that would be overlooked when using cDNA probes.

Based on the ease with which we can select α IMs against individual antibodies and their highly specific binding to the selecting antibody (Sullivan et al., submitted), we have adapted this technology to enrich for α IMs that appear to recognize antibody responses associated with RA. Starting with a library of only $\sim 2 \times 10^8$ sequence diversity, we were nevertheless able to identify a small number of probes that react well with at least two individual samples in a pool of 24. While this level of multisubject reactivity is modest, we expect that by using a library with much greater sequence diversity a larger variety of candidates would be recovered, and presumably yield clones with improved multisubject cross reactivity. Since we found 24 distinct sequences among the 80 candidates we analyzed and apart from the one abundant sequence present, nearly all of the remaining clones were unique isolates. This suggests that even within this pilot enrichment there are a substantial number of new sequences remaining to be characterized.

We employed a multivalent display system using the hyperphage [26] helper to produce phage displaying 3–5 copies of the α IM. We reasoned that the abundance of any one antibody in serum would be low, so we wanted to maximize the chances of recovering a bound phage. Multivalent display should ensure that a single phage could potentially bind to both arms of the captured IgG due to the presence of 3–5 copies of the α IM. While we have no direct confirmation of the efficacy of this strategy, it seemed prudent to provide the opportunity for bivalent binding to increase the probability of recovery of all possible binders, particularly for low abundance IgG specificities.

Most of our analysis has been conducted using the phage-displayed α IM probes in contrast to conventional cDNA or peptide probes that typically are synthesized and deposited on a slide or other solid phase for analysis of binding to serum antibodies. The α IM proteins are well suited for that purpose in that they are easy to express and purify, and as we

demonstrated for the 3–40 α IM, the use of the purified protein in a standard ELISA format yielded a reasonable alternative to the phage-based assay. However the conversion of large numbers of selected α IMs to soluble protein for immobilization is a less efficient process. The direct use of the phage-displayed α IM is conceptually appealing as it is a very low cost method for obtaining virtually unlimited amounts of the probe in a form that provides significant detection sensitivity due to the approximately 2800 copies of the major coat protein in the phage particle that is the target of anti-M13 antibody reagents.

An unexpected finding in this pilot study was the recovery of four distinct α IMs that bind to the IgG from the same subjects. We have observed when using individual monoclonal antibodies as targets, that some antibodies enrich a large number of binders from this library (>20), while others only a few (Sullivan et al., in preparation). Another potential bias influencing the selection in pooled serum is the likely uneven abundance of any particular disease-associated antibody. If any individual sample has a very high concentration of a particular antibody, this would be expected to focus the enrichment on α IMs that bind to the abundant target, and thereby make it more difficult to find α IMs to other antibody targets. This may be the explanation for the presence of a single clone in ~40% of our ELISA positive clones after three rounds of selection. A possible solution to these problems in future studies that the monobody technology is particularly well suited for is replacement of multiple rounds of enrichment and phage ELISA analysis with deep sequencing of the phage population after a single round of binding. Since the two variable loops in the monobody are only 185 nucleotides apart, this is well within the size range for effective sequencing of both loops with high-throughput sequencing technology [35,36]. Moreover, since it is straightforward to use two oligonucleotides designed directly from sequence information to re-create any α IM using site-directed mutagenesis, the entire phage screening process can potentially be eliminated and the number of candidate probes that can be characterized would be greatly increased. This offers a rapid pathway to comprehensively analyze a very diverse monobody library against many serum samples, whether pools or individuals, to detect sequences that are enriched and are correlated with disease status. Such an approach should have broad applicability and offers a common strategy for developing a robust diagnostic platform for diseases that lack adequate diagnostic tools.

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References

1. Cantaert T, De Rycke L, Bongartz T, Matteson EL, et al. Arthritis Rheum. 2006; 54:3381–3389. [PubMed: 17075816]
2. Koenig M, Fritzler MJ, Targoff IN, Troyanov Y, Senecal JL, L J. Arthritis Res Ther. 2007; 9:R78. [PubMed: 17688695]
3. Robert-Pachot M, Desbos A, Moreira A, Becchi M, et al. Autoimmunity. 2007; 40:380–389. [PubMed: 17612900]
4. Dinser R, Braun A, Jendro MC, Engel A. Scand J Rheumatol. 2007; 36:448–451. [PubMed: 18092266]
5. Bizzaro N. Autoimmun Rev. 2007; 6:325–333. [PubMed: 17537376]

6. Chatterjee M, Mohapatra S, Ionan A, G Bawa G, et al. *Cancer Res.* 2006; 66:1181–1190. [PubMed: 16424057]
7. Lin HS, Talwar HS, Tarca AL, Ionan A, Chatterjee M, et al. *Cancer Epidemiol Biomarkers Prev.* 2007; 16:2396–2405. [PubMed: 18006929]
8. Somers V, Govarts C, Somers K, Hupperts R, Medaer R, P Stinissen P. *J. Immunol.* 2008; 180:3957–3963. [PubMed: 18322204]
9. Kalnina Z, Silina K, Meistere I, Zayakin P, Rivosh A, et al. *J Immunol Methods.* 2008; 334:37–50. [PubMed: 18314130]
10. Papini AM. *J Pept Sci.* 2009; 15:621–8. [PubMed: 19714713]
11. Irving MB, Craig L, Menendez A, Gangadhar BP, Montero M, et al. *Mol Immunol.* 2010; 47:1137–1148. [PubMed: 20031219]
12. Koide A, Bailey CW, Huang X, Koide S. *J Mol Biol.* 1998; 284:1141–1151. [PubMed: 9837732]
13. Koide A, Koide S. *Methods Mol Biol.* 2007; 352:95–109. [PubMed: 17041261]
14. Barbas, CF., 3rd; Burton, DR.; Scott, JK.; Silverman, GJ. *Phage Display: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press; 2001.
15. Haidaris CG, Malone J, Sherrill LA, Bliss JM, M J, et al. *J Immunol Methods.* 2001; 257:185–202. [PubMed: 11687252]
16. Shea C, Bloedorn L L, Sullivan MA. *J Struct Funct Genomics.* 2005; 6:171–175. [PubMed: 16211515]
17. Barbas CF 3rd, Collet TA, Amberg W, Roben P, Binley JM, et al. *J Mol Biol.* 1993; 230:812–823. [PubMed: 8478936]
18. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, et al. *Science.* 2009; 326:285–289. [PubMed: 19729618]
19. Zwick MB, Jensen R, Church S, Wang M, Stiegler G, et al. *J Virol.* 2005; 79:1252–1261. [PubMed: 15613352]
20. Herold M, Boeser V, Russe E, Klotz W. *Clin Dev Immunol.* 2005; 12:131–135. [PubMed: 16050144]
21. Sebbag M, Simon M, Vincent C, Masson-Bessiere C, Girbal E, et al. *J Clin Invest.* 1995; 95:2672–2679. [PubMed: 7539459]
22. Girbal-Neuhauser E, Durieux JJ, Arnaud M, Dalbon P, M Sebbag M, et al. *J Immunol.* 1999; 162:585–594. [PubMed: 9886436]
23. DeJaco C, Klotz W, Larcher H, Duftner C, Schirmer M, M Herold M. *Arthritis Res Ther.* 2006; 8:R119. [PubMed: 16859519]
24. Kunkel TA, Roberts JD, Zakour RA. *Methods Enzymol.* 1987; 154:367–82. [PubMed: 3323813]
25. Tonikian R, Zhang Y, Boone C, Sidhu SS. *Nat Protoc.* 2007; 2:1368–1386. [PubMed: 17545975]
26. Broders O, Breitling F, Dubel S. *Methods Mol Biol.* 2003; 205:295–302. [PubMed: 12491895]
27. Zwick MB, Parren PW, Saphire EO, Church S, Wang M, et al. *J Virol.* 2003; 77:5863–5876. [PubMed: 12719580]
28. Zwick MB, Bonnycastle LL, Menendez A, Irving MB, et al. *J. Virol.* 2001; 75:6692–6699. [PubMed: 11413337]
29. Saphire EO, Montero M, Menendez A, van Houten NE, Irving MB, et al. *J Mol Biol.* 2007; 369:696–709. [PubMed: 17445828]
30. Leidinger P, Keller A, Ludwig N, Rheinhammer S, Hamacher J, et al. *Int J Cancer.* 2008; 123:1631–1636. [PubMed: 18649359]
31. Zhong L, Peng X, Hidalgo GE, Doherty DE, et al. *Proteomics.* 2004; 4 12-16-1225.
32. Siddiqui AH, Irani RA, Blackwell SC, Ramin SM, Kellems RE, Xia Y. *Hypertension.* 2010; 55:386–393. [PubMed: 19996068]
33. Cleutjens KB, Faber BC, Rousch M, van Doorn R, et al. *J Clin Invest.* 2008; 118:2979–2985. [PubMed: 18654662]
34. Oak S, Gilliam LK, Landin-Olsson M, Torn C, Kockum I, et al. *Proc Natl Acad Sci U S A.* 2008; 105:5471–5476. [PubMed: 18367670]
35. Metzker ML. *Nat Rev Genet.* 2010; 11:31–46. [PubMed: 19997069]

36. t'Hoen PAC, Jirka SMG, ten Broeke BR, Schultes EA, Aguilera B, Pang KH, et al. Analytical Biochem. 2012; 421:622–631.

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Figure 1. FN library construction strategy

(A) Structural representation of the fibronectin scaffold. β strands A–G of the FNfn10 molecular scaffold are shown, along with the 3 surface exposed loops (BC, FG and DE). The structure was generated using CN3D, from the source data file MMDB IB 57520. (B) Mutagenesis strategy for production of two-loop libraries. Stop template containing Ase I (ATTAAT) with in-frame TAA stop codons (underlined) is annealed to two library oligonucleotides that replace the BC and FG loop sequences with NHK codons while simultaneously eliminating the stop codons (and the Ase I restriction sites).

Phage specificity for wild-type b12 monoclonal antibody.

Figure 2. Specificity of a b12 α IM to Key Antigen Contact Residues of the b12 Antibody
 Mabs were produced by transient transfection of heavy and light expression plasmids into HEK293 cells. The Y53A, Y98A and W100A alanine substitutions were introduced into the b12 heavy chain plasmid and co-transfected with a b12 light chain plasmid and the antibodies were purified from the supernatant on protein G magnetic beads. The antibodies were captured on microtiter wells coated with goat anti-human IgG. The b12 binding α IM (3–5) and a negative control α IM (CRP) were as described (Sullivan et al., submitted). B2.1 is a positive control phage encoding a peptide mimotope that binds wild type and mutant forms of b12 [28]. Phage were prepared by polyethethylene glycol precipitation from 1.2 ml cultures, re-suspended in 0.3 ml in TBS+0.5% casein and 50 μ l was added to the wells. After 1 hour incubation at RT, the wells were washed 10 times and diluted anti-M13-Horseradish peroxidase (HRP) conjugate (GE Healthcare) was added for 1 hour. After washing, HRP substrate was added and after suitable color development, the reaction was quenched with acid and the absorbance was measured on a plate reader.

Figure 3. FN Library Enrichment on Individual Serum IgG

Capture wells were coated with antihuman IgG (15 $\mu\text{g/ml}$) and incubated with either subject serum (+) or pooled normal serum (-) using 1 $\mu\text{l/well}$. Phage were prepared from 16 individual clones from the second round of enrichment on single subject serum IgG were produced with hyperphage and applied to the wells. Wells coated with anti-Flag M2 Mab (Sigma) served as a control for phage production (shown only for the 1841 enrichment). After 1 hour incubation at RT, the wells were washed 10 times and diluted conjugate was added for 1 hour. After washing, HRP substrate was added and color was allowed to develop until suitable for photographing. The "X" indicates a no-phage production sample.

Figure 4. Binding analysis of clones from second and third round of enrichment on pooled positive serum IgG

Capture wells were coated with anti-human IgG and incubated with either pooled positive serum (PPS, +) or pooled normal serum (PNS, -) using 1 μ l/well. Phage were prepared from 16 individual clones from the second and third round of enrichment on pooled positive serum IgG were produced with hyperphage and applied to the wells. Detection and reaction development were as described in Figure 2.

Figure 5. Histogram of the reactivity of RA-specific clones to individual subject serum IgGs
Capture wells were coated with anti-human IgG (15 μ g/ml) and incubated with subject serum using 1 μ l/well in triplicate. Phage were prepared from four RA-reactive clones from the third round of enrichment on the pooled RA-positive samples with hyperphage along with phage prepared from vector only as a negative control for anti-phage reactivity in subject samples. Phage were prepared by PEG precipitation from 90 ml cultures, re-suspended in 6 ml of TBS+0.5% casein and 50 μ l was added to the wells. Detection and reaction development were as described in Figure 2, with the addition of phosphoric acid (0.5 M) to stop the HRP reaction. The absorbance of the plate was read at 620 nm and after subtraction of values from the control phage, the values were normalized to the well with maximum absorbance on the plate. The sequences of the BC and FG loops of each clone are shown below the histograms in single letter code.

Figure 6. Serum reactivity analysis using purified α IM protein

The 3–40 α IM protein ($\Delta, \blacktriangle$) along with an irrelevant negative control α IM ($\square, \blacksquare$) were prepared with biotin added *in vivo* near the carboxy-terminus and captured on streptavidin-coated immunoassay strips. Serum from a single patient (subject 10, $\Delta, \square$) reactive with the 3–40 α IM was diluted along with pooled normal serum ($\blacktriangle, \blacksquare$). Aliquots of dilutions were applied and bound IgG was detected with goat anti-human IgG-HRP as described in Methods.