Journal of Immunological Methods 391 (2013) 60–71

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Discovery of diverse and functional antibodies from large human repertoire antibody libraries

Lauren J. Schwimmer *, Betty Huang, Hoa Giang, Robyn L. Cotter, David S. Chemla-Vogel, Francis V. Dy, Eric M. Tam, Fangjiu Zhang, Pamela Toy, David J. Bohmann, Susan R. Watson, John W. Beaber, Nithin Reddy, Hua-Feng Kuan, Daniel H. Bedinger, Isaac J. Rondon

XOMA Corp., 2910 Seventh St., Berkeley, CA 94710, United States

ARTICLE INFO

Article history:
Received 23 October 2012
Received in revised form 12 December 2012
Accepted 21 February 2013
Available online 27 February 2013

Keywords:
Phage display
Antibody discovery
Human antibody repertoire

ABSTRACT

Phage display antibody libraries have a proven track record for the discovery of therapeutic human antibodies, increasing the demand for large and diverse phage antibody libraries for the discovery of new therapeutics. We have constructed naïve antibody phage display libraries in both Fab and scFv formats, with each library having more than 250 billion clones that encompass the human antibody repertoire. These libraries show high fidelity in open reading frame and expression percentages, and their V-gene family distribution, V_H-CDR3 length and amino acid usage mirror the natural diversity of human antibodies. Both the Fab and scFv libraries show robust sequence diversity in target-specific binders and differential V-gene usage for each target tested, supporting the use of libraries that utilize multiple display formats and V-gene utilization to maximize antibody-binding diversity. For each of the targets, clones with picomolar affinities were identified from at least one of the libraries and for the two targets assessed for activity, functional antibodies were identified from both libraries.

1. Introduction

Monoclonal antibodies are a significant and growing class of therapeutics for a wide range of indications including cancer, metabolic, and inflammatory diseases. Phage display antibody libraries are an important tool for the discovery of human monoclonal antibodies, providing two marketed products, one under review by the FDA, and many more at various stages of clinical trials (Nelson et al., 2010). Specificity and affinity are key components for the successful transition of an antibody from the lab to the clinic. Library size and diversity are extremely important in this endeavor as the larger and more diverse a library, the greater the chance of finding high affinity antibodies with diverse paratopes that bind diverse epitopes (Perelson and Oster, 1979; Perelson, 1989; Griffiths et al., 1994; Vaughan et al., 1996).

The first fully human phage displayed antibody fragment library had 10^7 members and antibody fragments to four proteins were isolated with affinities as low as 86 nM (Marks et al., 1991). Other groups went on to construct larger human libraries: two Fab (6.5×10^{10} and 3.7×10^{10}) (Griffiths et al., 1994; de Haard et al., 1999) and one scFv (1.4×10^{10}) (Vaughan et al., 1996). From each library, antibody fragments with single-digit nanomolar affinities were isolated, and from the scFv library, two fragments were isolated with affinities less than 1 nM. However, Fabs with only moderate affinities (>800 nM) were recovered when selecting from a small portion of the Griffiths library (10^7 clones), supporting the claim that the larger the library, the greater the probability of isolating high affinity antibodies (Griffiths et al., 1994). To

Abbreviations: scFv, single-chain fragment variable; Fab, fragment antigen binding; PBMC, peripheral blood mononuclear cell; V, variable; V_H, variable heavy chain; V_L, variable light chain; V_κ, variable kappa light chain; V_λ, variable lambda light chain; PPE, periplasmic extract; ORF, open reading frame; CDR, complementary determining region; FR, framework region; TIE, tyrosine kinase with immunoglobulin-like and EGF-like domains; β-gal, beta-galactosidase; ANG, angiopoeitin; Ins, insulin; InsR, insulin receptor; RCA, rolling circle amplification; HRP, horseradish peroxidase; PBS, phosphate buffered saline.

* Corresponding author. Tel.: +1 510 204 7200.
E-mail address: schwimmer@xoma.com (L.J. Schwimmer).

0022-1759
http://dx.doi.org/10.1016/j.jim.2013.02.010

this end, we constructed two phagemid libraries, XFab1 and XscFv2, which display Fab and scFv fragments, respectively, each with more than 2.5×10^{11} members maximizing the potential for isolating high affinity antibodies against any target of interest.

Antibody diversity is influenced by the number of donors, donor tissues used, the types of variable regions from which antibody sequences are amplified and the choice in the utilization of V-gene frameworks. For each of XFab1 and XscFv2, variable regions were amplified from thirty racially-diverse healthy donors using a variety of tissues including bone marrow, PBMCs, spleen and lymph node. The amplification strategy encompasses variable domains derived from IgM, IgG, IgA, IgE and IgD. While other commercial phage display antibody libraries have restricted antibody frameworks to enhance stability or expression of the displayed fragments (Söderlind et al., 2000; Hoet et al., 2005; Rothe et al., 2008), in the XFab1 and XscFv2 libraries, all prominent V-gene families encompassing the human repertoire were utilized to allow increased structural diversity. The performance of a Fab versus a scFv fragment type depends on factors including stability of the variable domains and the expression of the individual molecules (Rothlisberger et al., 2005), as well as the possible differences in the donor pools. Therefore, the performance characteristics of each library will differ, making it advantageous to have a variety of libraries available for selection. Although, fully human naïve Fab and scFv libraries have been made before (Marks et al., 1991; Griffiths et al., 1994; Vaughan et al., 1996; de Haard et al., 1999; Glanville et al., 2009; Lloyd et al., 2009), here we present the first direct comparison between the performances of the two formats. This comparison can be done because these two libraries were constructed using similar donor sources, construction methods and vector backbones, limiting the variability between the libraries.

Both XFab1 and XscFv2 were assessed for multiple qualification parameters, including percentage of open reading frame (%ORF), expression levels, V-gene family distribution, V_H-CDR3 length, and germline occurrence. Our libraries have been used for selections against seven targets and the resulting clones analyzed to determine unique hit rate, V-gene usage, and affinity. These parameters have allowed us to validate and compare the libraries and demonstrate their utility as potential sources for high affinity, functional therapeutic antibodies.

2. Materials and methods

2.1. Source material, cell lines, enzymes

The source RNA and cDNA used to amplify the V-genes was purchased from AllCells and Cureline. The *E. coli* strain TG1 (Lucigen) was used for all molecular cloning, phage production, and expression assays. Restriction endonucleases and T4-DNA ligase were purchased from New England Biolabs. KOD Hot Start DNA Polymerase and associated 10× buffer, dNTP mix, and $MgSO_4$ (EMD Biosciences), were used for all PCR reactions. Some PCR reactions also included betaine (Sigma-Aldrich) and/or DMSO (Sigma-Aldrich). PCR primers were purchased from Elim Biosciences or IDT. ArrayScript™ Reverse Transcriptase (Ambion) with Random primers (NEB) was used to make cDNA libraries from RNA samples. All media and solutions were purchased from Teknova.

For the CHO cells expressing TIE2 and InsR used for screening, mammalian expression vectors encoding TIE2 and InsR were each transfected into CHO-K1 cells using a PEI transfection reagent (JetPEI®, Polyplus). Individual G-418-resistant clones were screened by FACS using commercially available antibodies to TIE2 or InsR.

2.2. Library construction

2.2.1. XFab1

XFab1 used cDNA generated from 15 PBMC samples and 15 bone marrow samples. The variable regions were amplified from cDNA using primers designed based on sequences in V-Base to amplify each family of $V_\lambda 1$–$V_\lambda 10$, $V_\kappa 1$–$V_\kappa 6$, and $V_H 1$–$V_H 6$ individually with forward primers annealing to the V segment and reverse primers annealing in the C_λ or C_κ for V_λ and V_κ and in the V_HJ region for V_H (Table S1). Secondary PCRs (Table S2) were performed to add restriction enzyme sites 5′ and 3′ to each V-gene for cloning into pXHMV-US2-L-Fab or pXHMV-US2-K-Fab vectors (Fig. S1). The PCR products for each variable region were pooled according to the natural distribution as described on V-Base. The light chain variable regions were cloned first using restriction digest with *Sfi*I and *Avr*II for V_λ and *Sfi*I and *Bsi*WI for V_κ and transformed into electrocompetent TG1 cells (48 μg DNA in 48 200 μL transformations for V_κ and 65 μg DNA in 65 200 μL transformations for V_λ). Transformations were spread on 2xYT medium with 2% glucose and 100 μg/mL carbenicillin, which were incubated overnight at 30 °C. The following morning the bacteria were scraped from the plates, combined and plasmid DNA purified with the GenElute™ HP Maxiprep Kit (Sigma-Aldrich). The resulting DNA was prepared for cloning V_H with *Nco*I-HF and *Nhe*I-HF. The ligated DNA was cleaned with the Wizard® SV Gel and PCR Clean-up system (Promega) and transformed into electrocompetent TG1 cells (66 μg DNA in 66 200 μL transformations for V_κ and 100 μg DNA in 100 200 μL transformations for V_λ). Transformations were spread on 2xYT medium with 2% glucose and 100 μg/mL carbenicillin, which were incubated overnight at 30 °C. The following morning the bacteria were scraped from the plates, combined, and stored in 15% glycerol 2xYT at −80 °C.

2.2.2. XscFv2

The scFv library was constructed similarly to the above described Fab library with the following changes. Primer sequences are listed in Tables S3 and S4. cDNA from 20 PBMC samples, 8 bone marrow samples, 1 lymph node sample, and 1 spleen sample were used. The reverse secondary PCR primers for VH and forward secondary primers for V_κ and V_λ had complementary extensions for an AST(G_4S)3 linker and the forward secondary PCR primers for V_H and reverse secondary primers for V_κ and V_λ had sequences to add flanking *Sfi*I restriction sites. A tertiary PCR step was then done to assemble the full length scFv fragment, which was next cloned into pXHMV-scFv (Fig. S1) using the *Sfi*I sites. The ligated DNA was transformed into electrocompetent TG1 cells (147 μg DNA in 120 200 μL transformations for V_κ and 44 μg DNA in 40 200 μL transformations for V_λ). Transformations were spread on 2xYT medium with 2% glucose and

100 μg/mL carbenicillin, which were incubated overnight at 30 °C. The following morning the bacteria were scraped from the plates, combined, and stored in 15% glycerol 2xYT at −80 °C.

2.3. Phage rescue

Both XFab1 and XscFv2 phage libraries were rescued using a modification of the standard protocol (Marks et al., 1991). XFab1 was rescued in four batches (two for XFab1λ and two for XFab1κ) each starting with 5-fold more bacteria than the sub-library size. XscFv2 was rescued in five batches (two for XscFv2λ and three for XscFv2κ) with XscFv2λ starting 5-fold more bacteria than the sub-library size and XscFv2κ starting with 3.33-fold more bacteria than the sub-library size. For all rescue batches, cultures were seeded at a starting density of 0.1 OD_{600} in 2xYT medium with 2% glucose and 100 μg/mL carbenicillin and grown at 37 °C with shaking at 250 rpm until the OD_{600} reached 0.5. Half of the culture was then infected with 20 MOI M13KO7 and incubated at 37 °C for 1 h (30 min with no shaking and 30 min with shaking at 100 rpm). The culture was then centrifuged at 3000 RCF for 20 min. The pellets were resuspended in the same volume of 2xYT medium with 100 μg/mL carbenicillin and 50 μg/mL kanamycin. These cultures were grown 18 h at 25 °C with shaking at 250 rpm. Next, the cultures were centrifuged at 9000 RCF for 30 min and phage particles were purified from the supernatant by two PEG-precipitations (Sambrook and Russell, 2001). After the second precipitation, phage were resuspended in 1% of the initial volume with 15% glycerol in PBS and stored at −80 °C.

2.4. Selection of phagemid libraries

Selections against biotinylated gastrin 14-mer (Anaspec), β-galactosidase (Sigma), TIE-1-Fc chimera (R&D Systems), TIE-2 (R&D Systems), TIE-2/Ang2 (R&D Systems) and TIE-2/Ang1 (R&D Systems) were performed using solid or solution phase panning as previously described (Hawkins et al., 1992; Vaughan et al., 1996). Complexes of TIE-2 with Ang1 and Ang2 were formed in a 1:1 molar ratio prior to incubation with magnetic beads. Prior to panning, TIE1-Fc and TIE2 were biotinylated with the EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format (Thermo). InsR pannings were performed as previously described (Bhaskar et al., 2012).

2.5. Sequencing and sequence analysis

RCA sequencing was performed by either ELIM Biosciences or Sequetech. Sequences were analyzed for open reading frame (ORF), variable region family, and alignment to germline sequences. ORF and V-gene family were determined using SeqAgent™ (XOMA (US) LLC) following IMGT conventions. To determine percentage of germline representation in the naïve libraries and selected clones, V-Base germline DNA sequences were used as references. For each V-gene sequence, BLAST was used to find the closest germline match, followed by alignment of the two sequences using Clustalw2. The differences between the two sequences were then counted.

2.6. ELISA

Periplasmic extracts (PPE) of soluble scFvs and Fabs were prepared by growing 1 mL cultures of 2xYT medium with 0.1% glucose and 100 μg/mL carbenicillin to an OD_{600} of 0.5 at 37 °C with shaking in 96-well deep well plates. IPTG was then added to a final concentration of 1.25 mM and the cultures were grown 16 to 18 h at 30 °C with shaking. The cultures were pelleted and the supernatant removed. The pellets were resuspended in 75 μL PPB (Teknova) with protease inhibitors (Roche) and incubated for 10 min at 4 °C with shaking. Next, 225 μL of sterile water with protease inhibitors was added and incubated for 1 h at 4 °C with shaking. Cell debris was removed via centrifugation and the supernatant was removed as PPE.

Phage displaying scFv and Fabs were prepared by growing 1 mL cultures of 2xYT medium with 2% glucose and 100 μg/mL carbenicillin to an OD_{600} of 0.5 at 37 °C with shaking, usually in 96-well deep well plates. Next, 20 MOI of M13K07 helper phage was added to each well and allowed to infect for 1 h at 37 °C. After infection, the cultures were pelleted and resuspended in 1 mL 2xYT with 100 μg/mL carbenicillin and 50 μg/mL kanamycin and the cultures were then grown 16 to 18 h at 30 °C with shaking. Cells were removed via centrifugation and the supernatant was removed as phage.

For ELISA of PPEs, 96-well Maxisorp™ or Immulon-4 plates were coated with capture antibody (mouse anti-human IgG Fd (Millipore) for Fab or monoclonal anti-V5 (Sigma) for scFv) or antigen at 4 °C overnight. Plates were washed 3 times between each step with PBST (PBS + 0.05% Tween-20). Plates were blocked with either 5% milk or 10% casein in PBST for 1 h. After washing, PPEs were added to the plate and incubated for 1 h at room temperature. Plates were then washed and detection antibody was added (goat anti-human κ-HRPO (Invitrogen) or goat anti-human λ-HRPO (Invitrogen) for Fab, anti-His-HRP (Sigma) for scFv, or anti-V5 for antigen coated plates) and incubated for 1 h at room temperature. For antigen coated plates, after washing secondary antibody (goat α-mouse IgG (H + L), peroxidase conjugated (Thermo)) was added and incubated for 1 h at room temperature. Plates were then washed and HRP activity was detected with TMB Microwell Peroxidase Substrate System (KPL).

For ELISA of phage, 96-well Maxisorp™ or Immulon-4 plates were coated with capture antibody (goat anti-human κ (Invitrogen) or goat anti-human λ (Invitrogen) for Fab or monoclonal anti-V5 (Sigma) for scFv) at 4 °C overnight. Plates were washed 3 times between each step with PBST. Plates were blocked with either 5% milk or 10% casein in PBST for 1 h. After washing, phage were added to the plate and incubated for 1 h at room temperature. Plates were then washed and anti-M13-HRP antibody (GE Healthcare) was added and incubated for 1 h at room temperature. Plates were then washed and HRP activity was detected with TMB Microwell Peroxidase Substrate System (KPL).

2.7. Functional pAKT assays

CHOK1 cells engineered to express the TIE2 or InsR receptor were used. These cells were maintained in Growth Medium containing EX-CELL® 302 Serum-Free Medium for CHO Cells (Sigma-Aldrich), 2 mM L-glutamine, and 0.4 mg/mL

GENETICIN® (Invitrogen). On the day of the assay, the cells were washed and resuspended at 4×10^6 cells/mL in PBS with 0.5% BSA and incubated for 3 h at 37 °C, 5% CO_2 incubator. The test antibody or antigen was added for 10 min. For InsR + Ins, 375 pM insulin was added to the cells before incubation with antibody. After incubation, the treated cells were centrifuged and lysed in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich), and Complete Mini Protease Inhibitor (Roche Diagnostics Corporation) for 1 h with shaking at 4 °C. The lysates were clarified by centrifugation at 485 ×*g* for 3 min. The MULTI-SPOT Assay System (Meso Scale Discovery) was used to quantify the amount of phosphorylated AKT or MAPK present within the lysates. Data were analyzed using GraphPad Prism® (GraphPad Software Inc.) software.

2.8. K_D determinations

2.8.1. IgG capture chips

Monoclonal mouse anti-human IgG (Fc) antibody (GE Healthcare) was immobilized for capture on a Biacore A100 C1 Series S Biacore biosensor (GE Healthcare) by standard amine coupling. 100 to 200 RU of anti human IgG was immobilized on spots 1, 2, 4, and 5. Spot 3 was not used. Amine coupling was performed by activating the chip with EDC/NHS (GE Healthcare) for 10 min and injecting anti human IgG solutions at 2 μg/mL in pH5.0 acetate (GE Healthcare) for 7 min. Deactivation was performed with 1 M ethanolamine.

2.8.2. TIE2 IgG kinetics

TIE2 IgG were diluted to 1 μg/mL in assay running buffer and injected over anti human IgG for 4 min at 10 μL/min. TIE2 was injected over captured anti-TIE2 IgGs at five concentrations starting at 30 nM, four fold serial dilution created additional concentrations of 7.5 nM, 1.875 nM, 0.468 nM, and 0.117 nM. Injections were 4 min each at 30 μL/min in duplicate. Dissociation times were 5 min. Running buffer was HBS-EP (GE Healthcare) 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20 at pH 7.4 with 1 mg/mL BSA (Sigma). The capture surface was regenerated following each analyte injection with 3 M $MgCl_2$ (GE Healthcare).

2.8.3. Fab and scFv fragment capture chips

Antibody fragment capture surfaces were prepared on Biacore A100 CM5 Series S biosensors (GE Healthcare). Antibody was immobilized for capture on spots 1, 2, 4, and 5 by standard amine coupling. Spot 3 was not used. Fab capture utilized goat anti-human IgG (Fab specific) antibody (Jackson ImmunoResearch); scFv capture utilized monoclonal anti-6X histidine antibody (R&D Systems). Amine coupling was performed by activating the chip with EDC/NHS (GE Healthcare) for 10 min and injecting antibody solutions at 5 μg/mL in pH4.5 acetate (GE Healthcare) for another 10 min. Approximately 3000 to 4000 RU of goat anti-human IgG (Fab specific) antibody or 6000 to 8000 RU of anti-6X histidine antibody was immobilized. Deactivation was performed with 1 M ethanolamine.

2.8.4. Fab and scFv kinetics

Fab or scFv periplasmic extracts were diluted 1:1 in assay running buffer and filtered through 0.22 μm multiscreen GV filter plates (Millipore). Filtered periplasmic extracts were injected over anti-Fab IgG (for Fab PPE) or anti-6xHistidine IgG (for scFv) capture surfaces. TIE1 or β-gal was injected over captured Fab or scFv at two concentrations (100 nM and 50 nM for TIE1 and 100 nM and 25 nM for β-gal). Injections were 5 to 6 min each at 30 μL/min. Dissociation time was 15 min for TIE1 and 10 min for β-gal. Running buffer was HBS-EP (GE Healthcare) at pH 7.4 with 1 mg/mL BSA (Sigma). These assays conditions favor monomeric scFv (Desplancq et al., 1994; Arndt et al., 1998; Dolezal et al., 2000). The capture surface was regenerated following each analyte injection with 100 mM HCl. Data was double referenced by subtracting the reference spot within the flow cell which was an activated and deactivated blank surface as well as subtracting out blank (0 nM) injections. After referencing, the data were fitted to a 1:1 Langmuir interaction model using Biacore A100 evaluation software.

2.8.5. InsR affinity

Affinities of anti-InsR antibodies were determined as previously described (Rathanaswami et al., 2008). Briefly, the antibodies were incubated at a fixed 50 pM concentration with a titration series of human InsR expressing CHO-K1 cells at 5 °C for 18 h in PBS with 0.5% BSA and 0.1% sodium azide. Cells were removed by centrifugation and the amount of free antibody in the supernatant was measured by a sandwich immunoassay. Unbound antibody concentration data were curve-fit using KinExA™ software to yield the estimated affinity (K_D) values.

2.9. Screening by high-throughput flow cytometry for TIE2 or InsR binding

Suspension adapted CHO-K1 cells transfected with either human TIE1 or TIE2, and the parent cell-line were used in this assay. CHO-K1 cells were labeled with 600 nM CSFE (Invitrogen) and CHO-TIE1 cells were labeled with 100 nM CSFE (Invitrogen). Unlabelled CHO-TIE2 cells were mixed in equal numbers with the labeled TIE1 and CHO-K1 parent lines and the cell concentration adjusted to 2×10^6 cells/mL in FACS buffer (PBS (Life Technologies) with 0.5% BSA (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich)). Twenty-five microliters of the cell mixture was added to 25 μL of PPE and the suspension incubated at 4 °C for 60 min. The cells were then washed once with FACS buffer and the pellet resuspended in 25 μL of 1:1000 dilution of mouse anti-c-myc antibody (Roche) and incubated at 4 °C for 30 min. The cells were then washed once with FACS buffer and the pellet resuspended in 25 μL of 1:200 dilution of Alexa-647 conjugated goat anti-mouse antibody (Jackson ImmunoResearch) and incubated at 4 °C for 15 min. The cells were then washed once with FACS buffer and the pellet resuspended in 60 μL FACS buffer and analyzed on a FACScan (BD) modified by Cytek to have an AMS and Hudson plate crane. The resulting data were analyzed by FlowJo (Treestar) and Excel. Screening for PPEs that bind InsR was performed as previously described (Bhaskar et al., 2012).

3. Results

3.1. Fab and scFv library construction

The XFab1 and XscFv2 libraries were constructed using cDNA made from RNA isolated from bone marrow, PBMCs, spleens, or lymph nodes of thirty healthy donors for each library, with each library using different donors. The samples included RNA from at least 1×10^7 B-cells per library, therefore, accounting for random pairing of heavy and light V-genes, our theoretical maximum library size for each library was 1×10^{14}. This cDNA was used as a template with V-region specific primers (Tables S1–S4) to amplify the V_H, V_λ and V_κ regions of antibodies derived from the natural antibody repertoire, including IgM, IgG, IgD, IgA and IgE. For the XscFv2 library, all variable gene families annotated within V-Base (vbase.mrc-cpe.cam.ac.uk) were included in proportion to the theoretical human representation as described (Fig. 1). For XFab1, $V_\lambda 9$ and $V_H 7$ were not included due to restriction enzyme incompatibility and lack of amplification specificity, respectively.

For both libraries, V_λ and V_κ were independently cloned into phagemid vectors (Fig. S1) creating λ and κ sub-libraries, with XFab1κ (1.1×10^{11}) plus XFab1λ (1.4×10^{11}) having 2.6×10^{11} total members and XscFv2κ (2.8×10^{11}) plus XscFv2λ (8.2×10^{10}) having 3.6×10^{11} total members. Both vectors contain an amber stop codon between the antibody fragment and the phage gene 3, enabling soluble expression as well as display. Each antibody fragment (scFv or Fab V_H) is linked to a triple tag (6xHis, c-myc, and V5) to enable detection, capture and purification. The triple tag provides much needed flexibility, since many commercially available antigens utilize one or more of the individual tags above, disallowing their use in an assay with the antigen. Moreover, the V5 tag and 6xHis can be utilized simultaneously to capture and detect the soluble antibody fragment in an ELISA, allowing the determination of soluble antibody expression, as described below.

The percentage of clones with full length open reading frame (ORF) ranges from 66% to 85%. Between 58% and 85% of clones express soluble protein as assessed by ELISA (Table 1). Both libraries also have similar distributions of V_H-CDR3 lengths (Fig. 2) each with an average amino acid length of 15.3, which is similar to the distribution of V_H-CDR3 lengths of functional antibodies in the IMGT database (Giudicelli et al., 2006).

The V-genes from each library were also assessed for amino acid changes from germline sequences for FR1 through FR3 (Fig. 3A). Both libraries have similar average amino acid changes from germline sequences of less than two per segment in all but V_H-FR3. V_H-FR3 has greatest number of amino acid differences, averaging three amino acid differences per sequence. These differences are distributed throughout V_H-FR3, with no amino acid position contributing more to the diversity than others. Overall, the percentage of germline representation in the V-genes (FR1–FR3) ranges from 5.6% to 20.7% (Fig. 3B). The difference between the V_λ germline representation in XFab1 and XscFv2 can be accounted for by the difference in primers used to amplify these V-regions. For XscFv2, thirty-three primers were used to increase the specificity of the priming for each V_λ-gene family and subfamily over the eighteen primers used for V_λ priming for XFab1 (Tables S1 and S3). Since the primers were designed based on germline sequences, the result of having primers that are more specific is a decrease in natural diversity in FR1. To visualize more clearly the diversity of the libraries from germline sequences, Fig. 3C depicts the distribution of differences from germline sequences for each library. The majority of light chains have 5 or fewer differences from germline and the majority of heavy chains have 8 or fewer differences. For V_H, when there are more than twelve differences from germline, most of these differences are in FR3, which is reflected in the data presented in Fig. 3A. Whilst having antibody sequences as close to germline as possible may be desirable to avoid potential immunogenicity, mutations may be required to enhance affinity and/or specificity of antibodies for specific targets.

The pairing of heavy and light chain V-genes from each family occurs in proportion to their abundance in the library (data not shown), indicating random pairing as expected with the library construction method that was employed. Previous data suggests random pairing also occurs in the human repertoire (de Wildt et al., 1999).

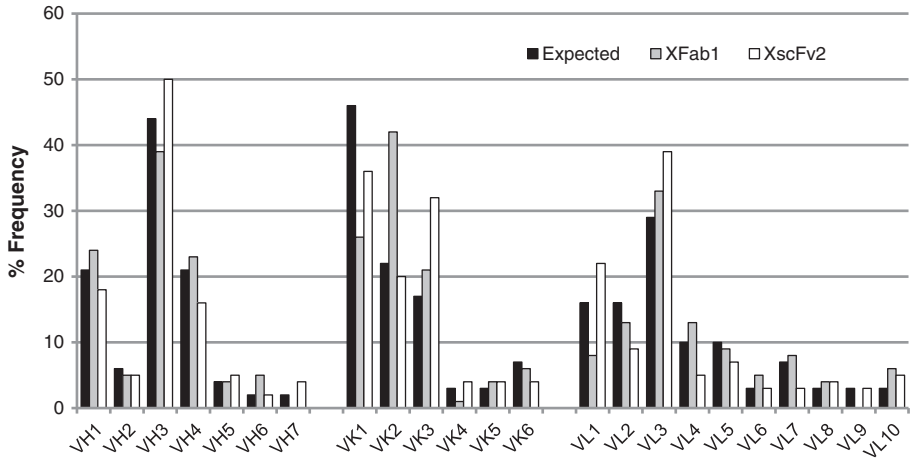

Fig. 1. Frequency of variable region families in the naïve XFab1 (gray) and XscFv2 (white) libraries compared to the expected frequency per VBase (black). Variable heavy (VH) family frequencies were calculated as a compilation of the V_κ (VK) and V_λ (VL) sub-libraries.

Table 1
Phage library characterization. Percentage open reading frame (%ORF) is calculated based on the number of sequences with complete open reading frame between start of signal sequences through the end of both the V_L and V_H for XFab1 and from the start of the signal sequence through the end of the VL for XscFv2. Percentage expression is based on the number of clones that had an OD_{450} >3-fold about background in an ELISA for periplasmic expression of Fab or scFv. The library size is expressed as colony forming units (cfus) from the transformation of the libraries.

	XFab1		XscFv2	
	V_κ	V_λ	V_κ	V_λ
% ORF	76	85	74	66
% Expression	70	85	71	58
Size	1.1×10^{11}	1.4×10^{11}	2.8×10^{11}	8.2×10^{10}

3.2. Selection and characterization of antigen binding antibody fragments

Each library was assessed by selection against seven targets: gastrin (a 14 amino acid peptide), β-galactosidase (a bacterial protein, β-gal), human proteins insulin receptor in complex with insulin (InsR + Ins), TIE1, TIE2, TIE2 in complex with angiopoeitin 1 (ANG1), and TIE2 in complex with angiopoeitin 2 (ANG2). Three rounds of panning were performed for each target using previously described panning methods (Hoet et al., 2005; Bhaskar et al., 2012). For each target, five to ten 96-well plates of clones were screened either by ELISA (gastrin, β-gal, TIE1, TIE2, and TIE2 complexes) or flow cytometry (InsR + Ins (Bhaskar et al., 2012), TIE2, and TIE2 complexes) for binding to the target. The clones that bound to their target were sequenced to identify unique clones. The unique clones were then analyzed for V_H and V_L family representation (Fig. 4), for CDR3 length of the V_H and V_L, and to assess the germline representation in FR1–FR3 of the selected clones (Table 2).

Once unique clones were identified for each target, further characterization of those clones was performed. For both libraries, the unique clones that bound to β-gal and TIE1 were prepared as soluble antibody fragments in periplasmic extracts (PPE) and the K_D (equilibrium dissociation constant)

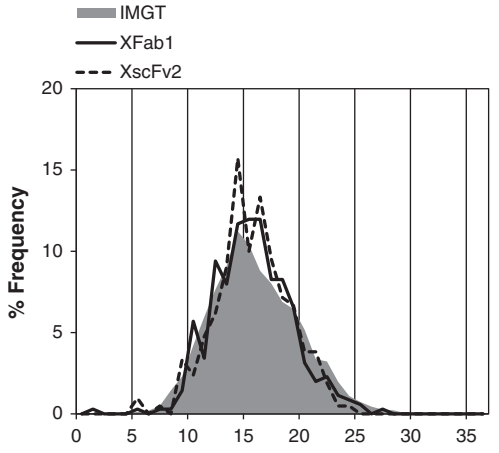

Fig. 2. Length distribution of V_H-CDR3 sequences from 6580 rearranged and productive human Ig-heavy cDNA or RNA sequences from the IMGT database (shaded), 351 naïve XFab1 (solid line), and 210 naïve XscFv2 (dashed line) sequences.

was determined using Biacore. For both targets, multiple antibody fragments with high affinities (single-digit nM to triple-digit pM) were identified. Table 2 lists the best K_D identified for each target per library (Fig. S3).

When screening the panning campaigns of TIE2 in complex with either of its ligands (ANG1 or ANG2), antibody fragments in PPE were screened by flow cytometry for binding to TIE2 or TIE2–ligand complex, and screened by ELISA for binding to ANG1 or ANG2. Binders in three categories emerged: single-protein binders (TIE2, ANG1 or ANG2), TIE2/ANG1-complex binders, or TIE2/ANG2-complex binders (Table 3). A subset of 10 Fab clones and 8 scFv clones that bound TIE2 was reformatted as IgG and the K_D for each clone was determined with Biacore (Table 2 and Fig. S3). For clones from XscFv2, 6 out of 8 clones have K_Ds > 8 nM. For clones from XFab1, 9 out of 10 have K_Ds > 11 nM with two of these clones having K_Ds in the pM range (Fab09 = 800 pM and Fab10 = 500 pM).

3.3. Analysis of selected clones

The sequences of the 591 unique selected clones for both libraries were compared to each other and aligned to the closest germline sequence. Of the 591 unique sequences that bound to the seven targets, there was no overlap in V_H-CDR3 sequences between those selected from XFab1 versus XscFv2, and the percentage of germline representation in the selected clones was comparable to that in the naïve library (Fig. 3B).

Next, the V-gene family usage of the selected clones was compared to that of the naïve library. We found that the family V-gene distribution of the selected clones (Fig. 4) was significantly different from the naïve library using a χ^2 test ($p < 0.002$ in all cases). Clones selected from XFab1 have more representatives from $V_\lambda4$–$V_\lambda10$ than those selected from XscFv2. In fact $V_\lambda5$, which makes up less than 5% of naïve XFab1, was over-represented in the selected Fab clones, with more than 20% of the clones having a light chain from this family. XscFv2 did not show this same preference for $V_\lambda5$. Also, families V_H2, V_H7, $V_\lambda9$, $V_\lambda4$, and $V_\kappa5$ were notably underrepresented in the selected clones of both libraries (note: V_H7 and $V_\lambda9$ were not included in XFab1). Target specific differences in V-gene usage were also evident. For example, despite the low representation of V_H5 in the bulk libraries (3–5%), 22% to 30% of the clones that bound to the InsR utilized V_H5 domain. There were also format specific, target dependent V-gene usage differences. For example, clones from the β-gal panning with XscFv2 had more V_H1 representation than V_H3, but with XFab1, V_H3 was more represented than V_H1. The opposite was seen in the InsR panning. We also noted that for all panning with XscFv2, more clones with kappa light chains were selected than those with lambda light chains, which may have been a reflection of the difference in size of the two sub-libraries (Table 1). In general, the selected clones from each library showed similar preferences for V_H–V_L family pairs. For selected clones from both libraries, V_H1 was often paired with $V_\lambda3$ and $V_\kappa1$, and V_H3 was often paired with $V_\lambda3$, $V_\kappa1$, $V_\kappa2$, and $V_\kappa3$. However there were some differences in family pair preference between the libraries for selected clones. For XscFv2, V_H1 was also often paired with $V_\kappa2$, and V_H3 with $V_\lambda1$, and $V_\kappa4$. For XFab1, V_H3 was also often paired with $V_\lambda5$.

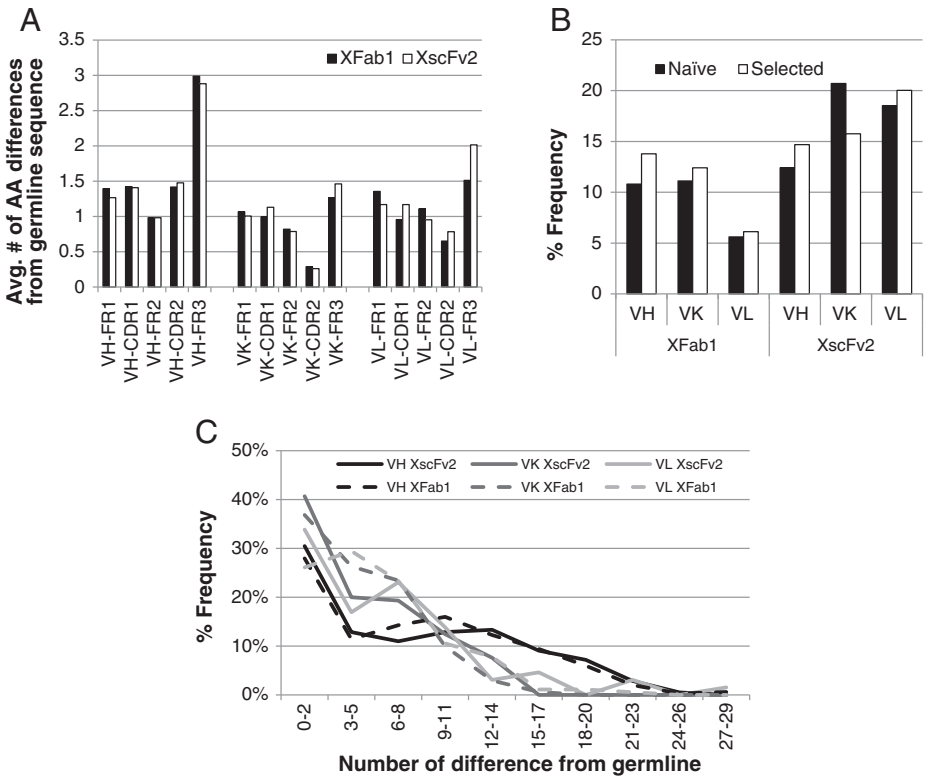

Fig. 3. A) Mean number of amino acid differences between naive XFab1 (black bars) or XscFv2 (white bars) sequences and germline antibody sequences in V_H, V_κ (VK) and V_λ (VL) frameworks (FR) 1–3 and complementary determining regions (CDR) 1 and 2. Germline sequences were obtained from VBase. B) Frequency of germline representation in V-genes (FR1through FR3) of the naïve libraries (black bars) and selected clones (white bars). For the selected clones, the value is the average percent frequency per target antigen. C) Frequency of amino acid differences from germline sequences in V_H (black lines), V_κ (VK) (dark gray lines) and V_λ (VL) (light gray lines) in FR1 through FR3 of XscFv2 (solid lines) and XFab1 (dashed lines).

3.4. Functional activity of phage display derived antibodies

To further validate these libraries, functional activity was assessed for two of the seven targets tested. Since ANG1/TIE2 interaction is known to mediate phosphorylation of Akt through PI3 kinase (DeBusk et al., 2004), IgG reformatted -TIE2 antibodies from XFab1 and XscFv2 were screened for their ability to promote Akt phosphorylation via activation of TIE2. Using the MesoScale Discovery Multi-spot Assay System, the phosphorylation level of serine 473 of Akt (pAKT) was measured after CHOK1 cells expressing human TIE2 on their surface were incubated with ANG1, -KLH (as a control) or the antibody of interest at 10 or 50 μg/mL. Eight of the eighteen antibodies tested induced AKT phosphorylation to at least 50% of the level induced by an endogenous agonist ANG1, with Fab05 (K_D = 7.8 nM) equaling the ANG1 pAKT level (Fig. 5).

The interaction between insulin and InsR also mediates phosphorylation of Akt at serine 473. Several high affinity antibodies for InsR selected from each library were also shown to be functionally active as IgG2s. Examples of three InsR agonist antibodies are shown in Fig. 6A. scFv20 activates InsR 27% relative to activation by insulin and has an affinity of 230 pM. Fab20 and Fab21, as IgG2s, activate InsR by greater than 50% with affinities of 24 pM and 27 pM, respectively.

In addition to agonists, positive and negative allosteric modulating antibodies were identified (Bhaskar et al., 2012) (Fig. 6B). The positive allosteric modulator, scFv21 as an IgG2, did not significantly activate InsR without insulin, however, in the presence of insulin there was a significant increase in AKT phosphorylation with the addition of antibody. As an IgG2, the affinity of scFv21 for InsR in the absence of insulin could not be determined because it bound so poorly, however, in the presence of insulin, the K_D was 1.6 nM (data not shown). For the negative allosteric modulator, scFv22, a decrease in AKT phosphorylation was seen with the addition of antibody (IgG2) in the presence of insulin. The binding affinity of scFv22 as an IgG2 (K_D = 50 pM) to InsR was the same in the presence or absence of insulin.

3.5. Amino acid usage in naïve and selected V_H-CDR3s

The V_H-CDR3 confers the majority of the contacts between the antibody and antigen, and is therefore a major contributor to the specificity and affinity of the antibody (Amit et al., 1986; Kabat and Wu, 1991). To analyze the amino acid usage of the two libraries, the V_H-CDR3 sequences of naïve and selected clones were compared to V_H-CDR3 sequences from 6580 productive human antibody sequences from the IMGT database (Giudicelli et al., 2006). While the amino acid distributions of the naïve and selected clones were, statistically, not the same as the IMGT distribution (χ^2 test, $p < 0.003$), the same general trends

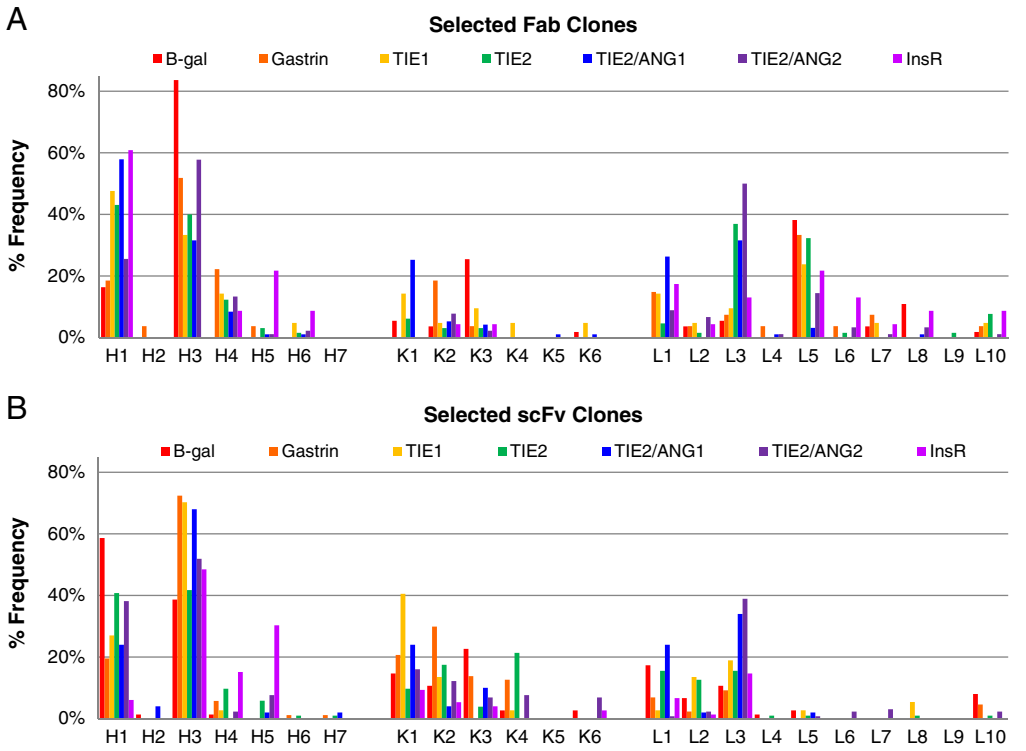

Fig. 4. Frequency of variable region families in the selected clones from XFab1 (A) and XscFv2 (B) libraries panned against seven target antigens — β-gal (red), gastrin (orange), TIE1 (yellow), TIE2 (green), TIE2/ANG1 (blue), TIE2/ANG2 (purple), InsR (magenta).

were observed as have been previously observed for human V_H-CDR3s (Zemlin et al., 2003) (Fig. 7). Notably, the selected clones appeared to have a greater percentage of tyrosine and glycine, but fewer cysteines than the naïve libraries or the antibodies in IMGT.

4. Discussion

The XFab1 and XscFv2 libraries were constructed to capture the diversity of the human antibody repertoire. Each library was generated from secondary lymphoid tissue from thirty

Table 2
Panning results summary. XFab1 and XscFv2 panning results against seven target antigens are characterized by the number of clones screened, the percentage of screened clones with >3-fold ELISA signal above background, the percentage of screened clones with >3-fold ELISA signal above background and unique amino acid sequences. The unique clones were also characterized by CDR3 length and aligned to germline antibody sequences. For some the the antigens, dissociation constants (K_D) were measured (see materials and methods). K_D for the clone with the highest affinity for its target listed here.

XFab1				Average Length		% Germline		
Target	Screened	% Positive	% Unique	V_H CDR3	V_L CDR3	VH	VL	K_D (nM)[a]
B-gal	744	25	7	11.6	8.2	6	13	1.0
Gastrin	465	33	5	12.1	8.4	4	13	ND
Tie1	744	10	3	15	8.4	9	5	2.1
Tie2	837	35	8	14.8	10.3	20	3	0.5
Tie2/Ang1	930	48	10	13.5	10.3	12	1	ND
Tie2/Ang2	837	28	11	15.8	10.5	23	4	ND
InsR	744	16	4	14.9	9.2	22	7	0.02
Average		28	7	14.0	9.3	14	7	
XscFv2								
B-gal	744	54	5	16.1	8.4	13	36	0.01
Gastrin	558	67	17	15	7.7	16	23	ND
Tie1	744	36	10	13.4	8	15	8	0.1
Tie2	930	62	11	13.9	9.8	18	15	1.5
Tie2/Ang1	930	85	5	13.8	10.2	14	18	ND
Tie2/Ang2	930	88	14	15	9.8	19	16	ND
InsR	744	16	7	13.4	8.7	22	14	0.23
Average		58	10	14.4	8.9	17	19	

[a] Lowest K_D measured.

Table 3
Panning against protein complexes. The distributions of antigen binding for antibody fragments that were selected from XFab1 or XscFv2 with panning done against TIE2/ANG1 or TIE2/ANG2 complexes are shown.

Library	Target	# Screened	TIE2	ANG1	ANG2	Complex
XFab1	TIE2/ANG1	837	6	56	NA	53
XFab1	TIE2/ANG2	837	23	NA	55	26
XscFv2	TIE2/ANG1	930	5	21	NA	26
XscFv2	TIE2/ANG2	930	41	NA	71	44

healthy donors, and both these libraries have more than 250 billion antibody fragments, making both libraries larger than any of the previously published libraries (Sblattero and Bradbury, 2000; Lloyd et al., 2009; Urlinger, 2011). These libraries encompass the full spectrum of V-gene families from IgM, IgG, IgE, IgA and IgD classes of immunoglobulins. The random pairing of V_H and V_L domains within and between donors increases the diversity by producing novel antibodies with heavy and light chain combinations that do not exist within the donor pool. The diversity of the V_H-CDR3 also mimics the human repertoire diversity in length and amino acid representation and distribution (Figs. 2, 7, and S2).

Upon selection, XFab1 and XscFv2 yield a high hit rate of unique antibody fragments which retain the diversity of the naïve libraries in V_H-CDR3 composition and germline representation. In the initial selections, XscFv2 yielded a higher percentage of clones that bound the target and a slightly higher percentage of unique clones than XFab1 (Table 2). However, more clones from XFab1 retain binding to the target upon reformatting to IgG than from XscFv2, so the yield of unique and functional clones from each library is typically balanced. Also, the retention of germline representation after selection allows the choice of a germline antibody for development, which may have less potential for immunogenicity.

Theoretically, the larger and more diverse an antibody library, the greater the probability of discovering a high affinity antibody for any target (Perelson and Oster, 1979; Perelson, 1989). According to Perelson, an antibody repertoire can be considered complete, having the ability to recognize any antigen, with only 10^5 members. However, just recognizing an antigen does not guarantee that the antibody will have the desired affinity or effect and increasing the repertoire size increases the probability of finding a high affinity antibody (Perelson, 1989). Griffiths and coworkers have demonstrated that a larger library yields higher affinity antibody fragments than a smaller subset of the same library (Griffiths et al., 1994). Here we demonstrated that with large antibody fragment libraries, XFab1 (2.5×10^{11}) and XscFv2 (3.6×10^{11}), antibodies and antibody fragments with picomolar affinities for multiple target antigens can be readily discovered (Table 2). For two targets we also performed functional assays and demonstrated that antibodies selected from these libraries are functional and are able to activate their target antigen. In addition to the antigens presented in this paper, these libraries were used for other therapeutic antibody programs. For those programs, antibodies with high affinity (<1 nM) and the desired function were discovered by screening fewer than 4000 clones and some with as few as 1000 clones screened. Also, for the majority of these programs affinity maturation will not be required.

The selected clones continued to represent the diverse populations from which they were selected. We continued to see a variety of V-gene families, although the distribution is different from that in the naïve libraries, and also varies according to target antigen (compare Figs. 1 and 4). Including all the prominent V-gene families in these libraries maximized the paratope diversity of the antibody fragments. The utilization of multiple V-gene families would not have evolved in the antibody generation process if they were not important for the function of the immune system and recognition of a multitude of antigens. One surprising example was the discovery by Sui and coworkers of broadly neutralizing anti-influenza activity of antibodies derived from the V_H1–69 germline family (Sui et al., 2009). The V_H1–69 family adopts a rare type-2 canonical structure CDRH2 loop, and consistently encodes two hydrophobic residues, including a unique germline Phe at the tip of the loop. Single framework phage display libraries (not built upon the V_H1–69 family) would have missed the unique structural reactivity provided by the V_H1–69 framework, thereby supporting use of the human repertoire of antibody frameworks in our libraries.

Sequence diversity in antibody frameworks is also important, as it directly affects the CDR loop conformation and orientation of V_H–V_L packing, thereby influencing the antibody paratope. V_H2, V_H4, and V_H6 families are predicted to adopt type 2 and type 3 canonical structures in CDRH1 and type 1 and type 5 canonical structures in CDRH2. In contrast, the major V-gene families V_H1 and V_H3 are predicted to adopt type 1 CDRH1 and primarily type 2, 3, and 4 CDRH2 loops (Vargas-Madrazo et al., 1997). Additionally, the V_H–V_L packing angle was better predicted when only framework residues were considered, suggesting that the influence of CDR residues on V_H–V_L orientation is small (Abhinandan and Martin, 2010). Antibody libraries that do not include the

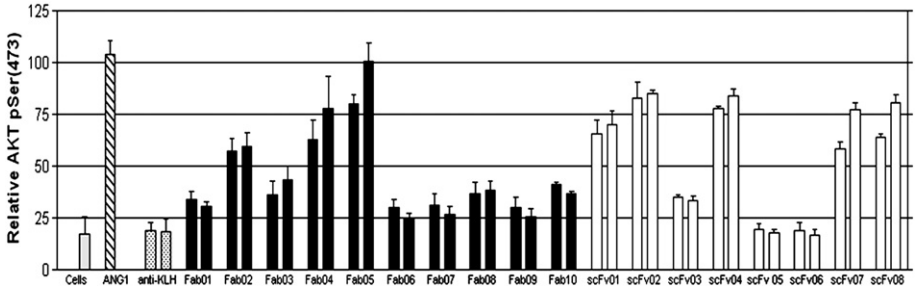

Fig. 5. Phosphorylation of AKT through activation of TIE2 by ANG1 or anti-TIE2 IgGs derived from XFab1 or XscFv2 with anti-KLH used as a negative control. Data are expressed as percentages of pAKT induced by 10 μg/mL of ANG1. For each pair, the left bar is 10 μg/mL antibody and the right bar is 50 μg/mL antibody.

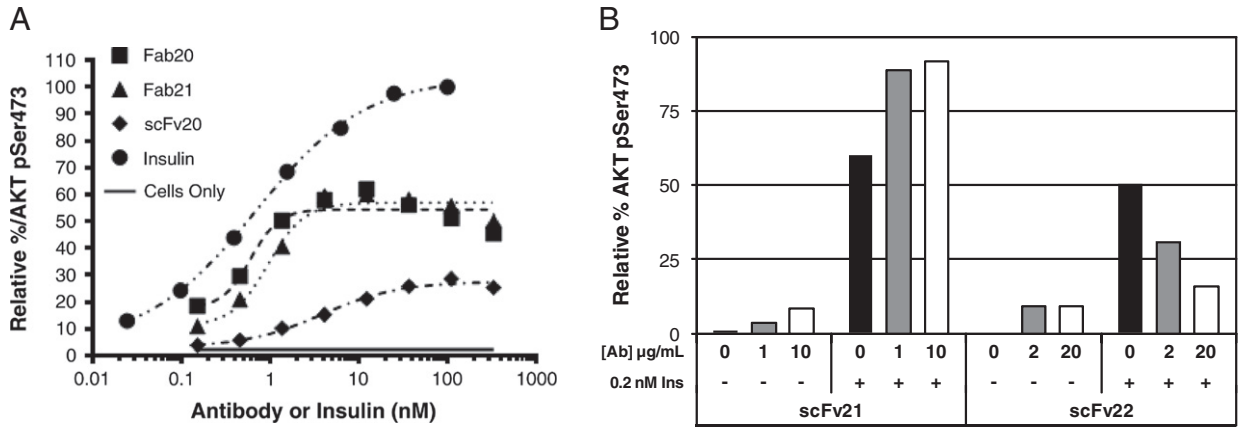

Fig. 6. A) Relative AKT phosphorylated at Ser473 in response to either increasing concentrations of insulin (circles), anti-InsR IgG2s (Fab20 — squares, Fab21 — triangles, scFv20 — diamonds), or vehicle (solid line). Data normalized so that 100 nM insulin is equal to 100% relative AKT phosphorylation. B) Relative AKT phosphorylation at Ser473 in response to treatment with either no antibody (left bar), 1 µg/mL scFv21 IgG2 or 2 µg/mL scFv22 IgG2 (middle bar), or 10 µg/mL scFv21 IgG2 or 20 µg/mL scFv22 IgG2 (right bar) for each set of three bars. For InsR + Ins, 0.2 nM insulin was added.

diversity encoded by the variable gene families are, therefore, limited in paratope diversity.

For the selections performed against InsR + Ins, antibody fragments with V_H5s were over-represented (Fig. 4). Interestingly, 64% of the negative allosteric InsR modulators (Fig. 6B, scFv226) utilize the V_H5 framework, whereas antibodies with other functions have no preference or favor the major V_H families, V_H1 and V_H3 (data not shown). Perhaps, this framework structure allows access to an InsR epitope not accessible by other frameworks.

Antibodies selected from XFab1 had greater representation from some of the minor V_λ families compared to antibodies selected from XscFv2. This was especially evident for V_λ5, which was vastly over-represented in the selected Fab clones (20%) versus its representation in the naïve XFab1 library (5%). It is known that the C_H–C_L heterodimer, which is not present in a scFv, contributes additional stability to the Fab fragment (Rothlisberger et al., 2005). Although, to our knowledge, an investigation of the stability of each V_L family has not been published, we hypothesize that the stabilizing effect of C_H–C_L allowed for selection of a wider variety of V_L families from the XFab1 library than the XscFv2 library. The preference for some V-gene families over others and the difference between the two formats may warrant further investigation of the stability and expression of each V-gene family in prokaryotes.

The diversity of the V_H-CDR3 amino acid sequences of the selected clones is particularly important as the V_H-CDR3 is the major contributor of contacts between the antibody and its antigen (Amit et al., 1986; Kabat and Wu, 1991). The

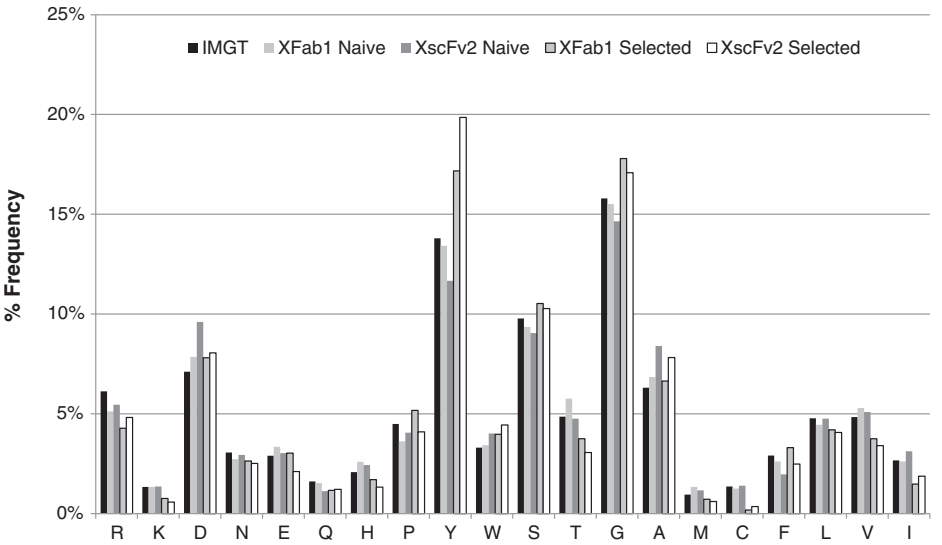

Fig. 7. Distribution of amino acid representation in V_H-CDR3 for amino acids 107 through 114 according to IMGT numbering. Single letter amino acid abbreviations are used on the x-axis and are in order of their hydrophobicity using the Kyte–Doolittle Index (Eisenberg, 1984).

distribution of amino acid sequences in this region of the clones selected from our libraries was similar to the naïve libraries and functional clones in the IMGT (Giudicelli et al., 2006) database (Figs. 7 and S2) and from previous analyses (Ivanov et al., 2002; Zemlin et al., 2003). The preferences for Tyr (for affinity (Fellouse et al., 2004; Birtalan et al., 2008)) and Gly (for flexibility (Mian et al., 1991; Padlan, 1994; Zemlin et al., 2003)) were especially evident in the clones selected from our libraries and were not unexpected since this amino acid preference is conserved across vertebrate species (Golub et al., 1997). On the other hand, Cys was under-represented in the selected clones. Where Cys did occur, it was in longer than average V_H-CDR3s and it occurred in pairs with three- to four-amino acid spacing. For these clones, disulfide bonded loops are likely to occur (Ramsland et al., 2001), probably adding stability to these loops. The Asp–Arg salt bridge that existed in approximately 60% of the selected clones may also contribute stability to the V_H-CDR3 (Zemlin et al., 2003).

We also demonstrated that in a single panning campaign it was possible to discover antibodies against multiple targets. After panning of TIE2 in combination with its ligand (either ANG1 or ANG2), antibody fragments that bound to TIE2 alone, ANG1 or ANG2 alone, or TIE2 in complex with ANG1 or ANG2 were recovered. The antibody fragments that bound only to the complexes of TIE2 are particularly interesting, and perhaps, were binding to new epitopes created in the complex formation.

In conclusion, we created two large and diverse antibody fragment phage display libraries to enable the discovery of therapeutic antibodies. From these libraries, functional clones with high affinity were selected for multiple antigens. The ability to select high affinity antibodies from these libraries minimizes the need for affinity maturation and allows researchers to focus on screening for clones with the desired binding properties and functionality.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2013.02.010.

Acknowledgments

We thank Mark White for his support and guidance throughout the library construction process. We also thank Toshihiko Takeuchi and John Corbin for lending technical support and for critical reading of this manuscript. The CHO-TIE1 and CHO-TIE2 cells lines used for screening and functional assays were created at XOMA by Genevieve Nonet and Rebecca Kaufman.

References

Abhinandan, K.R., Martin, A.C., 2010. Analysis and prediction of VH/VL packing in antibodies. Protein Eng. Des. Sel. 23, 689.

Amit, A.G., Mariuzza, R.A., Phillips, S.E., Poljak, R.J., 1986. Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution. Science 233, 747.

Arndt, K.M., Muller, K.M., Pluckthun, A., 1998. Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. Biochemistry 37, 12918.

Bhaskar, V., Goldfine, I.D., Bedinger, D.H., Lau, A., Kuan, H.F., Gross, L.M., Handa, M., Maddux, B.A., Watson, S.R., Zhu, S., Narasimha, A.J., Levy, R., Webster, L., Wijesuriya, S.D., Liu, N., Wu, X., Chemla-Vogel, D., Tran, C., Lee, S.R., Wong, S., Wilcock, D., White, M.L., Corbin, J.A., 2012. A fully human, allosteric monoclonal antibody that activates the insulin receptor and improves glycemic control. Diabetes 61, 1263–1271.

Birtalan, S., Zhang, Y., Fellouse, F.A., Shao, L., Schaefer, G., Sidhu, S.S., 2008. The intrinsic contributions of tyrosine, serine, glycine and arginine to the affinity and specificity of antibodies. J. Mol. Biol. 377, 1518.

de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J.W., Hoogenboom, H.R., 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem. 274, 18218.

de Wildt, R.M., Hoet, R.M., van Venrooij, W.J., Tomlinson, I.M., Winter, G., 1999. Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. J. Mol. Biol. 285, 895.

DeBusk, L.M., Hallahan, D.E., Lin, P.C., 2004. Akt is a major angiogenic mediator downstream of the Ang1/Tie2 signaling pathway. Exp. Cell Res. 298, 167.

Desplancq, D., King, D.J., Lawson, A.D., Mountain, A., 1994. Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. Protein Eng. 7, 1027.

Dolezal, O., Pearce, L.A., Lawrence, L.J., McCoy, A.J., Hudson, P.J., Kortt, A.A., 2000. ScFv multimers of the anti-neuraminidase antibody NC10: shortening of the linker in single-chain Fv fragment assembled in V(L) to V(H) orientation drives the formation of dimers, trimers, tetramers and higher molecular mass multimers. Protein Eng. 13, 565.

Eisenberg, D., 1984. Three-dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. 53, 595.

Fellouse, F.A., Wiesmann, C., Sidhu, S.S., 2004. Synthetic antibodies from a four-amino-acid code: a dominant role for tyrosine in antigen recognition. Proc. Natl. Acad. Sci. U. S. A. 101, 12467.

Giudicelli, V., Duroux, P., Ginestoux, C., Folch, G., Jabado-Michaloud, J., Chaume, D., Lefranc, M.P., 2006. IMGT/LIGM-DB, the IMGT comprehensive database of immunoglobulin and T cell receptor nucleotide sequences. Nucleic Acids Res. 34, D781.

Glanville, J., Zhai, W., Berka, J., Telman, D., Huerta, G., Mehta, G.R., Ni, I., Mei, L., Sundar, P.D., Day, G.M., Cox, D., Rajpal, A., Pons, J., 2009. Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. Proc. Natl. Acad. Sci. U. S. A. 106, 20216.

Golub, R., Fellah, J.S., Charlemagne, J., 1997. Structure and diversity of the heavy chain VDJ junctions in the developing Mexican axolotl. Immunogenetics 46, 402.

Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, R.E., Jones, P.T., Low, N.M., Allison, T.J., et al., 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. 13, 3245.

Hawkins, R.E., Russell, S.J., Winter, G., 1992. Selection of phage antibodies by binding affinity. Mimicking affinity maturation. J. Mol. Biol. 226, 889.

Hoet, R.M., Cohen, E.H., Kent, R.B., Rookey, K., Schoonbroodt, S., Hogan, S., Rem, L., Frans, N., Daukandt, M., Pieters, H., van Hegelsom, R., Neer, N.C., Nastri, H.G., Rondon, I.J., Leeds, J.A., Hufton, S.E., Huang, L., Kashin, I., Devlin, M., Kuang, G., Steukers, M., Viswanathan, M., Nixon, A.E., Sexton, D.J., Hoogenboom, H.R., Ladner, R.C., 2005. Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. Nat. Biotechnol. 23, 344.

Ivanov, I., Link, J., Ippolito, G.C., Schroder Jr., H.W., 2002. Constraints on the hydropathicity and sequence composition of HCDR3 are conserved across evolution. In: Zanetti, M., Capra, J.D. (Eds.), The Antibodies, vol. 7. Taylor & Francis, New York, p. 43.

Kabat, E.A., Wu, T.T., 1991. Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. Relative contributions of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. J. Immunol. 147, 1709.

Lloyd, C., Lowe, D., Edwards, B., Welsh, F., Dilks, T., Hardman, C., Vaughan, T., 2009. Modelling the human immune response: performance of a 1011 human antibody repertoire against a broad panel of therapeutically relevant antigens. Protein Eng. Des. Sel. 22, 159.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G., 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. 222, 581.

Mian, I.S., Bradwell, A.R., Olson, A.J., 1991. Structure, function and properties of antibody binding sites. J. Mol. Biol. 217, 133.

Nelson, A.L., Dhimolea, E., Reichert, J.M., 2010. Development trends for human monoclonal antibody therapeutics. Nat. Rev. Drug Discov. 9, 767.

Padlan, E.A., 1994. Anatomy of the antibody molecule. Mol. Immunol. 31, 169.

Perelson, A.S., 1989. Immune network theory. Immunol. Rev. 110, 5.

Perelson, A.S., Oster, G.F., 1979. Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. J. Theor. Biol. 81, 645.

Ramsland, P.A., Kaushik, A., Marchalonis, J.J., Edmundson, A.B., 2001. Incorporation of long CDR3s into V domains: implications for the structural evolution of the antibody-combining site. Exp. Clin. Immunogenet. 18, 176.

Rathanaswami, P., Babcook, J., Gallo, M., 2008. High-affinity binding measurements of antibodies to cell-surface-expressed antigens. Anal. Biochem. 373, 52.

Rothe, C., Urlinger, S., Lohning, C., Prassler, J., Stark, Y., Jager, U., Hubner, B., Bardroff, M., Pradel, I., Boss, M., Bittlingmaier, R., Bataa, T., Frisch, C., Brocks, B., Honegger, A., Urban, M., 2008. The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. J. Mol. Biol. 376, 1182.

Rothlisberger, D., Honegger, A., Pluckthun, A., 2005. Domain interactions in the Fab fragment: a comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of different stability. J. Mol. Biol. 347, 773.

Sambrook, J., Russell, D.W., 2001. Bacteriophage lambda and its vectors. In: Argentine, J. (Ed.), Molecular Cloning, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p. 2.45.

Sblattero, D., Bradbury, A., 2000. Exploiting recombination in single bacteria to make large phage antibody libraries. Nat. Biotechnol. 18, 75.

Söderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A.M., Nilsson, A., Jansson, B., Ohlin, M., Wingren, C., Danielsson, L., Carlsson, R., Borrebaeck, C.A., 2000. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. Nat. Biotechnol. 18, 852.

Sui, J., Hwang, W.C., Perez, S., Wei, G., Aird, D., Chen, L.M., Santelli, E., Stec, B., Cadwell, G., Ali, M., Wan, H., Murakami, A., Yammanuru, A., Han, T., Cox, N.J., Bankston, L.A., Donis, R.O., Liddington, R.C., Marasco, W.A., 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol. 16, 265.

Urlinger, S., 2011. Ylanthia: 100 billion high potentials. Antibody Engineering, San Diego, CA.

Vargas-Madrazo, E., Lara-Ochoa, F., Ramirez-Benites, M.C., Almagro, J.C., 1997. Evolution of the structural repertoire of the human V(H) and Vkappa germline genes. Int. Immunol. 9, 1801.

Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J., Johnson, K.S., 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat. Biotechnol. 14, 309.

Zemlin, M., Klinger, M., Link, J., Zemlin, C., Bauer, K., Engler, J.A., Schroeder Jr., H.W., Kirkham, P.M., 2003. Expressed murine and human CDR-H3 intervals of equal length exhibit distinct repertoires that differ in their amino acid composition and predicted range of structures. J. Mol. Biol. 334, 733.