A Highly Functional Synthetic Phage Display Library Containing over 40 Billion Human Antibody Clones

Marcel Weber1,2, Emil Bujak1,2, Alessia Putelli1,2, Alessandra Villa2, Mattia Matasci2, Laura Gualandi2, Teresa Hemmerle2, Sarah Wulhfard2, Dario Neri1*

1Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zürich, Zürich, Switzerland, 2Philochem AG, Otelfingen, Switzerland

Abstract

Several synthetic antibody phage display libraries have been created and used for the isolation of human monoclonal antibodies. The performance of antibody libraries, which is usually measured in terms of their ability to yield high-affinity binding specificities against target proteins of interest, depends both on technical aspects (such as library size and quality of cloning) and on design features (which influence the percentage of functional clones in the library and their ability to be used for practical applications). Here, we describe the design, construction and characterization of a combinatorial phage display library, comprising over 40 billion human antibody clones in single-chain fragment variable (scFv) format. The library was designed with the aim to obtain highly stable antibody clones, which can be affinity-purified on protein A supports, even when used in scFv format. The library was found to be highly functional, as >90% of randomly selected clones expressed the corresponding antibody. When selected against more than 15 antigens from various sources, the library always yielded specific and potent binders, at a higher frequency compared to previous antibody libraries. To demonstrate library performance in practical biomedical research projects, we isolated the human antibody GS, which reacts both against human and murine forms of the alternatively spliced BCD segment of tenascin-C, an extracellular matrix component frequently over-expressed in cancer and in chronic inflammation. The new library represents a useful source of binding specificities, both for academic research and for the development of antibody-based therapeutics.

Introduction

Monoclonal antibodies represent an important class of pharmaceutical biotechnology products and important research tools in chemistry and in life sciences [1,2]. The advent of phage display library technology [3–5] allowed the facile isolation of fully human antibodies from large combinatorial repertoires. While libraries were initially created starting from antibody genes isolated from natural sources (e.g., B cells in peripheral blood, spleen and tonsils [6,7]), there has been a growing interest in the construction of rationally designed synthetic antibody libraries, in which individual library members incorporate structural features which are beneficial for practical applications [8]. Such libraries may yield clones which are homogenous in terms of their biophysical properties and amino acid sequence (thus facilitating affinity maturation procedures [9]), with some additional desirable properties, such as protein A binding for affinity capture applications [10].

Over the last 15 years, we have described and extensively validated human antibody synthetic libraries, which featured antibodies in scFv format [11] capable of binding to protein A affinity supports [10]. These antibody libraries have been used as sources of useful binding specificities, including the monoclonal antibodies F8, L19 and F16, specific to the alternatively spliced EDA and EDB domain of fibronectin and the A1 domain of tenascin-C, respectively [9,12,13]. The three antibodies, which have been shown to selectively recognize stromal and neovascular structures in cancer [14,15] and inflammation [16], are able to preferentially localize at sites of pathological angiogenesis in vivo and are currently being investigated in Phase I and Phase II clinical trials [17–19].

In particular we have described antibody libraries of increasing size over the years: ETH2 (3×10^9 clones [13]), ETH2Gold (3×10^9 clones [20]), PHILO-1 and PHILO-2 (3.1×10^9 clones [21]), which were all based on the combinatorial randomization of judiciously selected amino acid residues in CDR3 loops of heavy and light chains, while keeping the rest of the antibody scaffold constant.

While the majority of the synthetic antibody libraries described so far are able to yield binders against the majority of the proteins chosen as targets, the ability to isolate various diverse antibodies in
a relatively short period of time (1–2 weeks) remains an important research goal in this field, in order to increase our ability to generate binding specificities against different epitopes and with different functional properties [21].

Here, we describe the design and construction of a very large antibody phage display library (termed “PHILODiamond”), containing over 40 billion human antibodies. This is the largest antibody library ever produced in our lab and one of the largest synthetic antibody libraries described in the literature [22]. The new library was highly functional, as revealed by the observation that >90% randomly picked antibody clones can be expressed at acceptable levels. A side-by-side comparison of antibody selections, performed with the PHILODiamond library and with other libraries against more than 15 antigens, revealed that various binding specificities could be isolated against structurally diverse targets.

The PHILODiamond library differs from other synthetic libraries in terms of size and modular design, facilitating affinity maturation procedures [12,23]. Furthermore, all antibody clones bind to protein A, thus facilitating purification and detection procedure [10]. We introduced a S52N mutation in the VH domain, since position 52 is the most frequently mutated solvent exposed residue in the CDR2 loop and since asparagine may favor both donor and acceptor hydrogen bonding interactions [21,24,25]. Position 52 is often mutated into an asparagine residue in naturally occurring antibodies [24,26].

In order to demonstrate library performance for biomedical research applications, we raised human monoclonal antibodies against the alternatively spliced BCD segment of tenasin-C, a highly conserved alternatively spliced protein fragment comprising the three fibronectin type-III homology domains B, C and D, which displays 86% sequence identity between mouse and man, respectively. The binding properties of clone G5, originating from the PHILODiamond library and with the randomized CDR3 part and

### Library Characterization

A total of 88 clones were tested by PCR screening with REDTaq Ready Mix PCR reaction mix (Sigma Aldrich) using primers [a] and [h] listed in Figure S1. Out of these clones, 55 were sequenced by Sanger sequencing (GATC Biotech) using primer [a] or [h]. For dot blot analysis, individual colonies from the plated library were inoculated in 160 μL 2xYT with ampicillin (100 μg/mL) and glucose (0.1%) in U-bottom 96-well plates (Nunc). The plates were incubated 4 h at 37°C in an orbital shaking incubator. Expression was induced by addition of 40 μL 2xYT containing IPTG to yield a final concentration of 1 mM and cultures were grown overnight at 30°C on an orbital shaker. ScFv-containing supernatants were blotted onto 0.45 μm nitrocellulose membrane (Santa Cruz Biotechnology) using the ELIFA system (Pierce). ScFv was detected with anti-myc tag murine antibody 9E10, followed by anti-mouse IgG horseradish peroxidase conjugate (Sigma Aldrich). Peroxidase activity was detected using the ECL plus western blotting detection system (Amersham Biosciences, GE Healthcare) on Amersham Hyperfilm ECL (Amersham Biosciences, GE Healthcare).

### Antibody Selection

Selections were performed according to standard protocols, using recombinant or purified antigen with high purity as assessed by SDS-PAGE and size exclusion chromatography. In brief, the antigen was either coated on MaxiSorp strips in 8 x 125 μL at around 5 x 10⁻⁶ M in PBS overnight, or the biotinylated antigen was coated on Streptawa (Roche) with 8 x 125 μL with a concentration of around 10⁻⁵ M or on 60 μL streptavidin-coated beads M280 (life technologies) in a volume of 200 μL for 30 min at RT. The wells or beads were blocked using 2% w/v skimmed milk powder in PBS (MPBS). After rinsing with PBS, about >10² phage particles were added to the antigen-coated surface in the presence of 2% MPBS, incubated for 2 h shaking (100 rpm) at RT. For the selections in wells after 30 min, the incubation was performed without agitation. Unbound phage were washed with PBS Tween 0.1% (7 to 20 times) and PBS (3 to 20 times), while bound phage were eluted with 100 mM triethylammonium (TEA). Eluted phage were neutralized by adding 1M Tris HCl pH 7.8 and used for infection of exponentially growing E. coli TG1. After 2 rounds of panning, ELISA screening was performed with 94 individual colonies as previously described by Silacci and colleagues [20]. In brief, individual colonies were inoculated in 200 μL 2xYT, 100 μg/mL ampicillin (Fisher Bioagents), 0.1% glucose (Sigma Aldrich) in Nunclon U-bottom 96-well plates (Nunc). The plates were incubated 3 h at 37°C in an orbital shaker...
Figure 1. Design and cloning strategy of the PHILODiamond antibody library. (A, B) Three-dimensional structure of a scFv antibody fragment, with randomized amino acids highlighted in circles. In the DP47 heavy chain fragment (in red) position 95–100 were randomly mutated and the length of the CDR varies from 4 to 7 amino acids. A point mutation at position 52, short before the CDR2, was introduced converting a Ser to an Asn, as marked with a star. The antibody in (A) shows the DPK22 light chain fragment (in blue), in which the Pro95 was kept constant and a Gly residue was allowed to be located at position 92 or 93. The antibody in (B) contains a light chain based on the DPL16 germline segment. The CDR3 of light chain contains at least one Pro residue, either at position 91, 92, 93, 94 or 95. (C) Cloning strategy for the construction of different sub-libraries, which were eventually pooled to yield the PHILODiamond library. Primers are listed in Figure S2. Two different cloning strategies were used. In the not-strategy, two DNA fragments coding for the heavy or light chain respectively were assembled and amplified using a primer containing the NotI-restriction site. In the abc-strategy, an additional DNA segment, which contained the NotI-restriction site, was assembled together with the randomized light and heavy chain fragments. (D) All DNA fragments were amplified, double digested and ligated into the pHEN1 phagemid vector. All numbers in the antibody sequences are according to Tomlinson et al [43]. The three-dimensional structures were modelled from the Protein Data Bank files 1IGM (DPK22) and 8FAB (DPL16).

doi:10.1371/journal.pone.0100000.g001
Expression and Purification of ScFv

A single colony was used to inoculate 10 ml of 2xYT media containing ampicillin (100 µg/mL) and 1% glucose and were incubated at 37°C on an orbital shaker until they were dense (about an absorbance at OD600 nm of 1). This preculture was diluted 1:100 in 400 mL of 2xYT containing ampicillin and 0.1% glucose and grown at 37°C until the OD600 nm reached 0.4. The cells were than induced by addition of IPTG to a final concentration of 1 mM and grown over night at 30°C. The scFv antibody fragments were purified over Protein A Agarose (Sino Biotechnology) and eluted with 100 mM triethylamine.

Size-Exclusion Chromatography (SEC)

Purified antibody fragments were analyzed on ÄKTA FPLC (GE Healthcare) using a Superdex 75 10/300 GL or Superdex 200 10/300 GL column, for a scFv or small immune protein (SIP) antibody fragment, respectively. For further analysis, the monomeric fraction was collected.

Surface Plasmon Resonance (SPR) Analysis

SPR was performed on a Biacore 3000 system (Biacore, GE Healthcare) using a CM3, CM5 or SA chip (Biacore) coated with the desired protein at 10 μL/min flow rate. 20–30 μL of antibodies were injected at different concentrations. Regeneration of the chip was performed by injecting 5 μL of 10 mM HCl. For supernatant screening, the same chips were used, and 20 μL the filtered (0.22 μm) supernatant were injected.

Expression of BCD Domain of TnC

Recombinant murine BCD domains were cloned into pQE12 vector, expressed in E. coli and purified over NiNTA resin as described earlier [28–30] for human BCD domain, the sequence was cloned into pUC119 vector, an additional AVI-Tag followed

Table 1. Results of selections performed with the PHILODiamond library and with other libraries.

| Antigen                  | PHILODiamond | ETH2 Gold | PHILO1–2 |
|--------------------------|--------------|-----------|----------|
|                          | ROP Positive | ROP Positive | ROP Positive |
| Fibronectin (7B89)       | 2            | 48        | N.P.     |
| Collagen                 | 2            | 1         | 2         |
| Fibrinogen               | 2            | 26        | N.P.     |
| Follistatin-like protein 1 | 2          | 7         | N.P.     |
| Glutathione-S-transferase | 2          | 61        | 2         |
| Tenascin-C (BCD)         | 2            | 30        | 2         |
| Human matrix metalloproteinase 1 (MMP1) | 2 | 16 | N.P. |
| Human matrix metalloproteinase 3 (MMP3) | 2 | 18 | 23 |
| Mycolactone              | 2            | 2         | N.P.     |
| Proteasome 26S (PSMD6)   | 2            | 14        | 2         |
| Pentraxin-related protein 3 | 2          | 14        | N.P.     |
| Serpin                   | 2            | 4         | 2         |
| Tissue inhibitor of metalloproteinase (TIMP) | 2 | 57 | 23 |
| Ubiquilin 1 (UBQL1)      | 2            | 12        | N.P.     |
| Translocase of the outer membrane (TOM) | 3 | 3 | 3 |

In the left column, antigens that were used for selections are listed in alphabetical order. After 2 or 3 rounds of panning (ROP), 94 bacterial colonies were grown, induced in 200 μL 2xYT media and checked by ELISA for the presence of antigen-specific scFv fragments. The number of positive clones (out of the 94 colonies screened) corresponds to ELISA signals, which were higher than the signal of TG1 cell supernatant (negative control) by at least 20-fold. N.P.: not performed.

doi:10.1371/journal.pone.0100000.t001
by the His-Tag was fused at the C-terminus of the protein, and the fusion protein was expressed in E. coli.

Reformatting into SIP (Small Immune Protein) Format
The DNA of the scFv was assembled with the CH4-DNA of a human IgE and cloned into a pcDNA3.1 vector (Invitrogen) as described earlier [12,23,31]. Expression of the protein was performed as previously described [32] using transient gene expression (TGE). Six days after transfection, the SIP proteins were purified from the supernatant by affinity chromatography using Protein A Agarose (Sino Biological).

Immunofluorescence on Tumor Section
Xenograft or murine tumors were excised, embedded in NEG-50 freezing medium (Thermo Scientific) and stored at −80°C until sectioning. An immunofluorescence analysis on tumor sections (10 μm) was performed. The slides were fixed for 10 min in ice-cold acetone, rehydrated with PBS and blocked for 1 h with fetal calf serum (FCS). The slides were incubated with SIP antibodies (250 nM in PBS - 1% BSA) for 1 h at RT. The staining of primary antibodies was detected using an anti-human IgE antibody produced in rabbit and in a next step an anti-rabbit Alexa Fluor 549 (each 10 μg/ml in PBS - 1% BSA) (Invitrogen) for 30 min at RT. Nuclei were stained with DAPI (Invitrogen) and vessels were stained using a rat anti-CD31 antibody and an anti-rat Alexa Fluor 488 conjugate (not shown in the pictures). Each step was followed by 3 washes with PBS. Slides were mounted with Fluorescent Mounting medium (Dako), and images were acquired with a Zeiss Axioskop 2 MOT Plus (Carl Zeiss AG). Image analysis was performed using AxioVision 4.7 image analysis software (Carl Zeiss AG).

Ethics Statement about Animal Sections
All murine and xenografts used for this analysis were prepared on the basis of our Project License (42/2012) at ETH Zurich. The License was issued by the Veterinaeramt des Kanton Zuerich, to the name of Dario Neri.

Results
Design and Cloning of the Antibody Library
To generate a large, stable and highly diverse library of functional antibody in the single chain variable fragment (scFv) format, we cloned scFv fragments with sequence diversity restricted to the CDR3 loops of both heavy and light chain into a phagemid vector. A cloning strategy based on PCR-assembly steps was adopted (Figure 1), which resulted in a total number of 4.16 × 10^10 independent clones. We used human antibodies based on the scFv format, with the DP47 germline sequence for the heavy chain variable domain, which confers high thermal stability and protein A binding properties [10]. In full analogy to previous antibody libraries, we chose either DPK22 or DPL16 as germline genes for the light chain and we used the flexible polypeptide linker GGGGSGGGGSGGGG to connect VH and VL in the scFv antibody format [20,21]. The DP47, DPK22 and DPL16 germline genes are frequently used in humans, representing 12, 25 and 16% of the antibody repertoire, respectively [33]. CDR3 loops in VH domains were allowed to contain 4, 5, 6 or 7 combinatorial mutated amino acids, while CDR3 loops in VL domains were randomized in 5 or 6 positions (Figure 1). Furthermore, residue 52 of VH domains was designed to be an asparagine, in order to facilitate hydrogen bonding interactions. The complete amino acid sequence of the designed antibody library can be found in Figure S1.

The functionality of the library was initially verified by sequencing randomly picked clones, by studying the frequency of antibody-expressing clones and by PCR analysis of insert size.
All unselected and sequenced clones (n = 55) showed a different CDR3 region in both heavy and the light chains. In dot-blot analysis, we found that more than 90% of the unselected clones were able to express scFv fragments, which could be detected with the anti-myc tag 9E10 antibody. PCR screening analysis revealed that 93% of the clones contained an insert of the right size (Figure 2 and Figure S2).

Antibody Selections

The PHILODiamond library was screened against a panel of more than 15 proteins, yielding positive clones against every target antigen, often with strongly positive clones already after two rounds of panning (Table 1). Most of the binding antibody fragments were further analyzed by sequencing, gel-filtration and by surface plasmon resonance (SPR) analysis for KD determination. Some SPR-profiles are shown in Figure 3. KD values typically ranged between 9 nM and 150 nM when measured with monomeric scFv fragment preparations, isolated after 2 or 3 rounds of panning. No obvious correlation could be observed between enrichment frequency, ELISA signal intensity and Biacore performance. Typically, scFv fragments may form non-covalent homodimeric structures (“diabodies”), contributing to functional binding affinity [34]. The distribution of lengths of randomized positions in VH CDR3 loops found in antibodies after antigen selection is displayed in Figure 4. The distribution reveals a preference for longer CDRs (six combinatorially mutated amino acids), compared to the results obtained with a synthetic library of similar design (ETH2Gold), previously reported by our group [20]. In CDR3 loops of VL domains, most binders had a preference for proline at position 95 or 96 of the randomized segment (Figure S3).

Selection and Characterization of a Novel Monoclonal Antibody Specific to the Alternatively Spliced BCD Segment of Tenascin-C

In order to confirm that the PHILODiamond library was able to yield high-quality binders against proteins of pharmaceutical interest, we selected an antibody (termed G5) against the alternatively spliced BCD segment of tenascin-C. This fragment, which exhibits a 86% amino acid identity between mouse and man, is frequently over-expressed in cancer and in chronic inflammatory conditions [35], while being virtually undetectable in normal adult tissues [9].

The G5 antibody clone was isolated after two rounds of panning against a biotinylated version of recombinantly expressed murine BCD antigen [9] and reacted strongly with both murine and human cognate antigen. The complete amino acid sequence of the antibody clone is reported in Figure S1. The binding properties of the G5 clone in monomeric scFv format and in homodimeric SIP format [12,36] against the human and the murine isoforms of BCD were analyzed by surface plasmon resonance on a Biacore instrument (Figure 5). The monomeric scFv antibody preparation exhibited similar kinetic binding constants towards the human and the murine isoforms of BCD were analyzed by surface plasmon resonance on a Biacore chip (Figure 5).

The ability of the G5 antibody to recognize the cognate antigen in normal and tumoral tissue, from both mouse and human origin, was assessed using immunofluorescence staining procedures on
angiogenesis and of tumor stroma [29], which was studied in more addition, the library yielded G5, an antibody fragment specific to alternatively spliced BCD segment of tenascin-C, a marker of fibronectin and A1 domain of F16, two clinical-stage human antibodies specific to the alternatively spliced EDA domain of fibronectin and A1 domain of tenascin-C, respectively [9,12–16,19,37,38]. G5 revealed a stain-
ting properties of the G5 antibody were studied by immunofluo-
sections of freshly frozen specimens. G5 was compared to F8 and F16, two clinical-stage human antibodies specific to the alternatively spliced EDA domain of fibronectin, which recognizes the cognate antigen in mouse and man and as a negative control KSF (specific to hen-egg lysozyme). All antibodies were tested in SIP format. doi:10.1371/journal.pone.0100000.g006

sections of freshly frozen specimens. G5 was compared to F8 and F16, two clinical-stage human antibodies specific to the alternatively spliced EDA domain of fibronectin and A1 domain of tenascin-C, respectively [9,12–16,19,37,38]. G5 revealed a staining pattern similar to the one of F16, with a strong reactivity against A375 (human malignant melanoma), MDA-MB 231 (human mammary gland), SK-RC-52 (human renal cell carcinoma) and U87 (human glioblastoma) tumors (Figure 6).

Discussion

There is a growing interest in the use of antibody phage technology for the generation of fully human monoclonal antibodies. Phage display libraries differ in terms of antibody sequence, germline usage, randomization strategy, as well as library size and functionality. In this paper, we described a highly functional synthetic antibody displaying phage display library, containing over 40 billion clones. The library design incorporated germline sequences, which are often found in the human antibody repertoire and which were previously successfully incorporated in smaller but highly functional phage display libraries [20,21]. The PHILODiamond library yielded specific binders against all protein antigens used as target (Table 1), including clones with KD values <10 nM originating directly from library selections. As a representative, few SPR profiles are shown in Figure 3. In addition, the library yielded G5, an antibody fragment specific to the alternatively spliced BCD segment of tenascin-C, a marker of angiogenesis and of tumor stroma [29], which was studied in more detail, because of its possible biomedical applications. The antibody was found to bind human and mouse antigen with comparable affinity (Figure 5) and to strongly react with various types of tumors. By contrast, G5 did not stain virtually all normal adult tissues tested, exception made for eccrine sweat glands in skin and a weak staining in small intestine (Figure S4).

Synthetic naïve libraries are based on antibody genes, which are randomized at defined positions, while immunized libraries are based on VH and VL domains derived from an animal’s immune repertoire. Synthetic antibody libraries tend to yield clones with more homogenous properties and to perform better against highly conserved antigens, since antibody genes have not undergone in vivo negative selection [39]. We chose to concentrate amino acid diversity in the CDR3 loops of heavy and light chains, since these positions are frequently involved in contact with the antigen [40–42]. However, we also chose asparagine as residue 52 of VH, as this position is frequently changed during somatic hypermutation [41,43] and in affinity maturation procedures [44]. Library clones based on the DPL16 germline gene, we inserted at least one proline at positions 91, 92, 93, 95 or 96, according to previously published strategies [20,21]. Sequence analysis of PHILODiamond-derived clones revealed a preference for a proline insertion at position 95 or 96, which may favor beta turn formation.

The DP47 germline VH gene, chosen for library construction, presents a number of attractive features. First, it is frequently associated with high thermal stability of the corresponding antibody clones [45]. Second, it confers binding to protein A even in scFv format, a feature, which is particularly attractive for antibody purification and for immuno-detection purposes [10,23,46]. The PHILODiamond library was found to contain >90% of functional clones (Figure 2 and Figure S2). It was tested on more than 15 antigens, ranging from big size molecules like collagen-I or fibrinogen, over a broad range of targets, including small catalytic domains (e.g., MMP3, TIMP or GST) and small toxic molecules (e.g., mycolactone) (Table 1).

The modular design of the library allows a facile reformattting of antibody clones in several functional variants, such as SIPS or full length IgGs [36] Furthermore, the concentration of amino acid diversity in CDR3 loops facilitates the implementation of affinity maturation procedures by randomization of CDR1 or CDR2 region, as recently shown for the selection of antibodies against Placental Alkaline Phosphatase, an ovarian cancer marker [23].

We chose to use the scFv antibody rather than Fab fragments or dAbs, as scFv’s tend to express better and yield higher levels of antibody display on filamentous phage. On the other hand, scFv fragments may form non-covalent oligomers, a feature which is not shared by Fab fragments. Antibody clones based on scFv fragments can be easily reformatted into intact human immuno-globulins [31,36], while the same feature is not possible with dAb-based antibodies, which lack the light chain domain.

The PHILODiamond library performed better for most antigens (e.g. GST, PSMD6 or TIMP), or at least in a similar fashion (e.g. for BCD, Serpin or Mycolactone), when compared in side-by-side selections with the ETH2-Gold library. Only in the case of collagen I, fewer antibody clones were isolated from the PHILODiamond library. The new library presents a number of attractive features, including binding to protein A for all library members. This property cannot be achieved using other synthetic libraries (e.g., [47]), which make use of various types of germline genes coding for the VH domain.

Tenascin-C is an extracellular matrix component, which exists in various splice isoforms. While the extra-domains A to D are absent in the small tenascin-C isoform, which is found in several healthy tissues, splice isoforms containing extra-domains exhibit a
more restricted pattern of expression in normal organs. By contrast, large tenascin-C isoforms can be very abundant during embryogenesis, in cancer and in chronic inflammation [9,16,19,25,33,48,49]. The G5 antibody recognizes its cognate antigen in mouse and human specimens, with prominent stromal and vascular pattern of staining. As such, it is ideally suited for the development of antibody-based targeted biopharmaceuticals, which may carry cytotoxic drugs [50–53], radionuclides [54] or cytokines [55] as therapeutic payloads. Our group has recently reported promising examples of therapeutic activity in cancer patients for anti-tenascin-C antibodies, armed with interleukin-2 [19,56] or with iodine-131 [57].

In summary, we have described a large and highly functional synthetic phage display library, which may be broadly useful for the isolation of antigen-binding specificities. The technology has been perfected over the years, to an extent that virtually any purified protein can be successfully used as target for antibody selections. The performance of the anti-tenascin G5 antibody was shown that affinity reagents, directly isolated from the library, perform well for biomedical research applications.

Supporting Information

Figure S1 Used primers and sequence of SIP G5. (A) Primers used for the library construction. (B) Full length sequence of the anti-BCD antibody G5 in the SIP format. (EPS)

Figure S2 Quality control of the six sub-libraries. (A) Dot blot analysis of 94 induced supernatants of individual unselected library clones. The soluble scFv were detected with an anti-myc-tag antibody (9E10) and a secondary antibody coupled with HRP. Positive controls are marked with a green circle. A red circle surrounds negative controls. The number below the library name represents the titer of individual clones in the sub-library. (B) Agarose gel of the PCR colony screening of unselected clones using primers annealing up- and down-stream of the scFv gene inserted into the pHEN1 vector. The number below the library names represents the number of clones carrying the right insert compared to total number of screened clones. (EPS)

Figure S3 Analysis of the light chains of well-characterized antibodies with good affinity against different antigens. (A) According to the library design it is possible that a glycinne is present at position 2 (1), at position 3 (5) or neither positions 2 or 3 (4). Some clones had more than a single glycinne inside CDR3. At position 5 there is always a Pro. (B) The CDR3 of the DPL16 has a glycine at position 5. (EPS)

Figure S4 Immunofluorescence of G5 anti-BCD antibody tested on a frozen tissue array from healthy humans (BioChain, T6234700-5, B403108). G5 antibody is shown in green, nuclei are stained with DAPI (shown in blue). (EPS)

Acknowledgments

The authors are grateful to Ásta Rós Sítnýggösdóttir for her help in protein expression.

Author Contributions

Conceived and designed the experiments: MW AV MM LG TH. Analyzed the data: MW EB AP MM LG SW DN. Performed the experiments: MW EB AP MM LG TH. Provided reagents/materials/analysis tools: MW EB AP MM LG SW TH DN. Wrote the paper: MW DN.

References

1. Slikowskii MX, Meffin J (2013) Antibody therapeutics in cancer. Science 341: 1192–1198.
2. Russ NA, Henderson SJ, McFarlane M, Shenton JM, de Haan L (2012) Monoclonal antibody therapeutics: history and future. Curr Opin Pharmacol 12: 615–622.
3. Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228: 1315–1317.
4. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. Annu Rev Immunol 12: 433–455.
5. Lerner RA, Bartley AS, Rabin JD, Burtn DR, Barbas CF, 3rd (1992) Antibodies without immunization. Science 258: 1313–1314.
6. Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1993) Making antibody fragments using phage display libraries. Nature 362: 624–628.
7. Merz DC, Dunn RJ, Drapeau P (1995) Generating a phage display antibody library against an identified neuron. J Neurosci Methods 62: 213–219.
8. Marks JD, Griffiths AD, Malmqvist M, Clackson TP, Byrne JM, et al. (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. Biotechnology (N Y) 10: 779–783.
9. Brack SS, Silacci M, Birchler M, Neri D (2006) Tumor-targeting properties of synthetic phage display libraries. Nature 352: 624–628.
10. Hoogenboom HR, Winter G (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J Mol Biol 227: 381–388.
11. Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc Natl Acad Sci U S A 85: 5679–5683.
12. Villa A, Trachsel E, Kaspar M, Schliemann C, Sommavilla R, et al. (2008) A new epitope of oncofetal fibronectin. MAbs 3: 264–272.
13. Pini A, Viti F, Santucci A, Carrinella B, Zardi L, et al. (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. J Biol Chem 273: 21769–21776.
14. Schliemann C, Widmer A, Pedretti M, Sacrezpanowski M, Klöpper W, et al. (2009) Three clinical-stage tumor targeting antibodies reveal differential expression of oncofetal fibronectin and tenasin-C isoforms in human lymphoma. Leuk Res 33: 1716–1722.
15. Schliemann C, Neri D, Rosli C, Neri D, Rosli-Khabas M, et al. (2011) A comparative immunofluorescence analysis of three clinical-stage antibodies in head and neck cancer. Head Neck Oncol 3: 25.
16. Schliemann C, Kaspar M, Bootz F, Marcelongu R, Paresce E, et al. (2009) Preclinical characterization of DEKAVIL (F9-IL16), a novel clinical-stage immunocytokine which inhibits the progression of collagen-induced arthritis. Arthritis Res Ther 11: R142.
17. Schliemann C, Neri D (2007) Antibody-based targeting of the tumor vasculature. Biochim Biophys Acta 1776: 173–192.
18. Schliemann C, Neri D (2010) Antibody-based vascular tumor targeting. Recent Results Cancer Res 180: 201–216.
19. Gutbrodt KL, Schliemann C, Giovannoni L, Frey K, Pahat T, et al. (2013) Antibody-based delivery of interleukin-2 to neovascularature has potent activity against acute myeloid leukaemia. Sci Transl Med 5: 201ra118.
20. Silacci M, Brack S, Schirru G, Marinald J, Ettorre A, et al. (2005) Design, construction, and characterization of a large synthetic human antibody phage display library. Proteomics 5: 2549–2550.
21. Villa A, Lovato V, Bujak E, Wullbrandt P, Pasche N, et al. (2011) A novel synthetic naive human antibody library allows the isolation of antibodies against a new epitope of oncofetal fibronectin. MAbs 3: 264–272.
22. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K (2011) High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. Molecules 16: 3673–3700.
23. Ravenni N, Weber M, Neri D (2013) A human monoclonal antibody specific to placental alkaline phosphatase, a marker of ovarian cancer. MAbs 6.
24. Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G (1992) The repertoire of human germine VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J Mol Biol 227: 776–798.
25. Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G (1992) The repertoire of human germine VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J Mol Biol 227: 776–798.
26. Chothia C, Lesk AM, Gherardi E, Tomlinson IM, Walter G, et al. (1992) Structural repertoire of the human VH segments. J Mol Biol 225: 799–817.
27. Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, et al. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19: 4133–4137.

28. Balza E, Siri A, Ponassi M, Casco F, Linnala A, et al. (1993) Production and characterization of monoclonal antibodies specific for different epitopes of human tenascin. FEBS Lett 332: 39–43.

29. Carnevallo B, Castellani P, Ponassi M, Borsì L, Urbini S, et al. (1996) Identification of a glioblastoma-associated tenascin-C isoform by a high affinity recombinant antibody. Am J Pathol 145: 1345–1352.

30. Silacci M, Brack SS, Spath N, Buck A, Hillinger S, et al. (2006) Human monoclonal antibodies to domain C of tenascin-C selectively target solid tumors in vivo. Protein Eng Des Sel 19: 471–478.

31. Borsì L, Balza E, Bestagno M, Castellani P, Carnevallo B, et al. (2002) Selective targeting of tumoral vascularization: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. Int J Cancer 102: 75–85.

32. Lit T, Neri D (2012) Biodistribution studies with tumor-targeting bispecific antibodies reveal selective accumulation at the tumor site. Mabs 4: 775–783.

33. Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 13: 3245–3250.

34. Holliger P, Hudson PJ (2005) Engineered antibody fragments and the rise of single domains. Nat Biotechnol 23: 1126–1136.

35. Midwood KS, Hassenet T, Langlois B, Orend G (2011) Advances in tenascin-C biology. Cell Mol Life Sci 68: 3175–3199.

36. Zuberbühler K, Palumbo A, Bacci C, Giovannoni L, Sommavilla R, et al. (2009) A general method for the selection of high-level scFv and IgG antibody expression by stably transfected mammalian cells. Protein Eng Des Sel 22: 169–174.

37. Pedretti M, Verpelli C, Marlind J, Bertani G, Sala C, et al. (2010) Combination of temozolomide with immunocytokine F16-IL2 for the treatment of glioblastoma. Br J Cancer 103: 827–836.

38. Schwager K, Bootz F, Imesch P, Kaspar M, Trachsel E, et al. (2011) The syngeogeneic mouse model. Hum Reprod 26: 2344–2352.

39. Pedretti M, Rancie Z, Soltermann A, Herzog BA, Schliemann C, et al. (2010) Comparative immunohistochemical staining of atherosclerotic plaques using F16, F8 and L19: Three clinical-grade fully human antibodies. Atherosclerosis 208: 382–389.

40. Hoogenboom HR, Marks JD, Griffiths AD, Winter G (1992) Building antibodies from their genes. Immunol Rev 130: 41–59.

41. Rothe C, Urdinger S, Lohning C, Prasler J, Stark Y, et al. (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: 1182–1200.

42. Ma X, Barthelemy PA, Rouge L, Wiesmann C, Sirlin SS (2013) Design of synthetic autonomous VH domain libraries and structural analysis of a VH domain bound to vascular endothelial growth factor. J Mol Biol 425: 2247–2259.

43. Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 13: 3245–3250.

44. Rothlisberger D, Honegger A, Plückthun 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: 773–783.

45. Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, et al. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19: 4133–4137.

46. Marks JD, Bradbury A (2004) General methods for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19: 5703–5710.

47. Al-Lazikani B, Lesk AM, Chothia C (1997) Standard conformations for the hypervariable regions of immunoglobulin light chains: a comparative analysis of the framework conformations. EMBO J 16: 348–359.

48. Balza E, Siri A, Ponassi M, Casco F, Linnala A, et al. (1993) Production and characterization of monoclonal antibodies specific for different epitopes of human tenascin. FEBS Lett 332: 39–43.

49. Carnevallo B, Castellani P, Ponassi M, Borsì L, Urbini S, et al. (1996) Identification of a glioblastoma-associated tenascin-C isoform by a high affinity recombinant antibody. Am J Pathol 145: 1345–1352.

50. Silacci M, Brack SS, Spath N, Buck A, Hillinger S, et al. (2006) Human monoclonal antibodies to domain C of tenascin-C selectively target solid tumors in vivo. Protein Eng Des Sel 19: 471–478.

51. Borsì L, Balza E, Bestagno M, Castellani P, Carnevallo B, et al. (2002) Selective targeting of tumoral vascularization: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. Int J Cancer 102: 75–85.

52. Lit T, Neri D (2012) Biodistribution studies with tumor-targeting bispecific antibodies reveal selective accumulation at the tumor site. Mabs 4: 775–783.

53. Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J 13: 3245–3250.

54. Holliger P, Hudson PJ (2005) Engineered antibody fragments and the rise of single domains. Nat Biotechnol 23: 1126–1136.

55. Midwood KS, Hassenet T, Langlois B, Orend G (2011) Advances in tenascin-C biology. Cell Mol Life Sci 68: 3175–3199.

56. Zuberbühler K, Palumbo A, Bacci C, Giovannoni L, Sommavilla R, et al. (2009) A general method for the selection of high-level scFv and IgG antibody expression by stably transfected mammalian cells. Protein Eng Des Sel 22: 169–174.

57. Pedretti M, Verpelli C, Marlind J, Bertani G, Sala C, et al. (2010) Combination of temozolomide with immunocytokine F16-IL2 for the treatment of glioblastoma. Br J Cancer 103: 827–836.

58. Schwager K, Bootz F, Imesch P, Kaspar M, Trachsel E, et al. (2011) The antibody-mediated targeted delivery of interferon-α10 inhibits endothroietin in a syngeneic mouse model. Hum Reprod 26: 2344–2352.

59. Marks JD, Bradbury A (2004) Selection of human antibodies from phage display libraries. Methods Mol Biol 248: 161–176.

60. Chothia C, Lesk AM (1995) The structural repertoire of the human V kappa domain. EMBO J 14: 4628–4638.

61. Ma X, Barthelemy PA, Rouge L, Wiesmann C, Sirlin SS (2013) Design of synthetic autonomous VH domain libraries and structural analysis of a VH domain bound to vascular endothelial growth factor. J Mol Biol 425: 2247–2259.

62. Rothlisberger D, Honegger A, Plückthun 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: 773–783.