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A novel synthetic naïve human antibody library allows the isolation of antibodies against a new epitope of oncofetal fibronectin

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Human monoclonal antibodies (mAbs) can routinely be isolated from phage display libraries against virtually any protein available in sufficient purity and quantity, but library design can influence epitope coverage on the target antigen. Here we describe the construction of a novel synthetic human antibody phage display library that incorporates hydrophilic or charged residues at position 52 of the CDR2 loop of the variable heavy chain domain, instead of the serine residue found in the corresponding germline gene. The novel library was used to isolate human mAbs to various antigens, including the alternatively-spliced EDA domain of fibronectin, a marker of tumor angiogenesis. In particular, the mAb 2H7 was proven to bind to a novel epitope on EDA, which does not overlap with the one recognized by the clinical-stage F8 antibody. F8 and 2H7 were used for the construction of chelating recombinant antibodies (CRAbs), whose tumor-targeting properties were assessed in vivo in biodistribution studies in mice bearing F9 teratocarcinoma, revealing a preferential accumulation at the tumor site.

Introduction

Human monoclonal antibodies (mAbs) and their derivatives represent the largest and fastest growing sector of pharmaceutical biotechnology products.[1,2] Antibody phage display libraries are useful tools for the isolation of human mAbs for therapeutic and in vivo diagnostic applications.[3] Indeed, good-quality binders can be obtained in virtually all cases from selection experiments, whenever the corresponding antigen is available as pure protein in 1–2 milligram quantities.[4-11]

Adalimumab (Humira®) was the first mAb isolated using phage library technology to be approved for therapeutic applications in Europe, the US and many other countries.[12] At present, several phage-derived antibodies are being investigated in clinical trials. Our group has isolated human antibodies against components of the modified extracellular matrix that are currently being investigated in clinical trials either as radiolabeled products or as cytokine fusion proteins.[13,14] In particular, the F8 antibody, which is specific to the alternatively-spliced extra domain (EDA) of fibronectin,[15] the L19 antibody (specific to the alternatively-spliced EDB of fibronectin[16-18]) and the F16 antibody (specific to the alternatively-spliced A1 domain of tenascin-C[19]) have exhibited an impressive potential to selectively localize at neovascular structures in solid tumors and in lymphomas.[20] The three antibodies were isolated from the ETH2-GOLD synthetic antibody library[11,21] and affinity-matured using a procedure featuring the combinatorial mutagenesis of residues in CDR1 and CDR2 loops and stringent selections on immobilized antigen. Indeed, the ETH2-GOLD library had been constructed using the most-commonly used VH germline segment (DP47,[22]) and the frequently used Vκ and Vλ germline segments DPK22 and DPL16,[23,24] with combinatorial mutagenesis only for residues of the CDR3 loops of heavy and light chain.[11]

Over the years, the ETH2-GOLD library has proven to be a reliable source of good-quality human mAbs.[11,15,19,25-32] However, as for other libraries, a degree of epitope bias may result from the library design. For example, we were so far unable to isolate further human mAbs from the ETH2-GOLD library that do not overlap with the clinical-stage F8 antibody. We reasoned that the construction of a synthetic antibody library, which closely resembled the design of ETH2-GOLD but which would incorporate charged or hydrophilic residues at central positions of the antigen binding site, could be used for the isolation of mAbs to novel epitopes in target proteins of interest. Indeed, a similar strategy had previously been used to drive antibody selections against certain epitopes on target antigens with defined arrangements of charged residues on their surface.[33]

Here, we describe the design and construction of a novel antibody phage display library (termed PHILO library) that was used for the isolation of good-quality binding specificities against a variety of different antigens. Interestingly, the PHILO library allowed the isolation of human antibodies specific to an epitope

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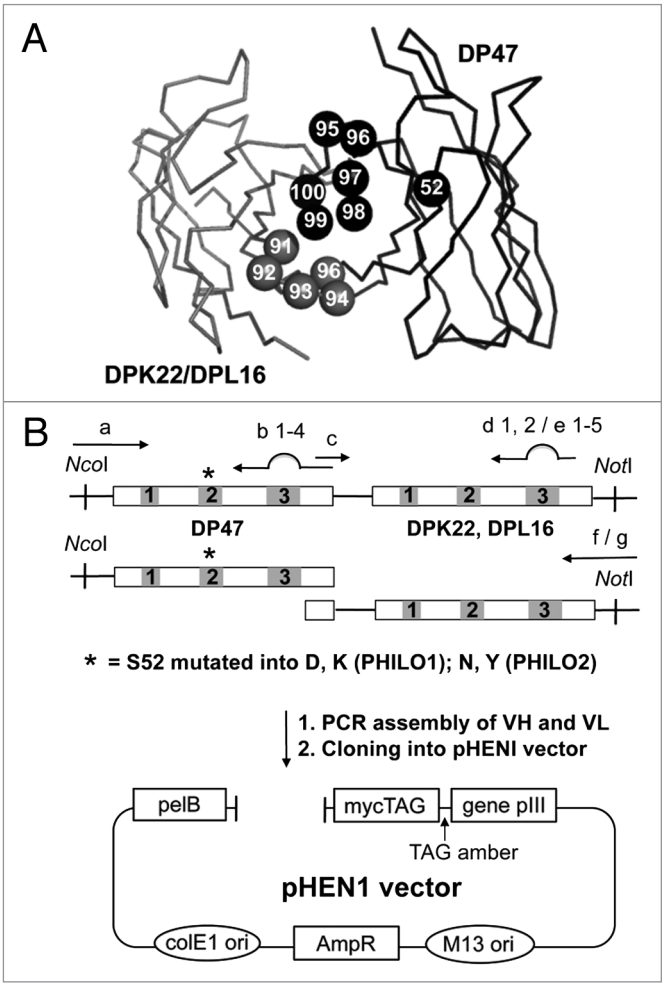

Figure 1. Design and cloning strategy for the PHILO antibody library. (A) Three-dimensional structure of a scFv antibody fragment. Heavy chain and light chain backbone are represented in dark grey and light grey, respectively. Residues subject to random mutation are DP47 CDR3 position 95–100 (dark grey space-fill representation), light chain position 91 to 96 (light grey space-fill representation) (in more details, for DPK22 CDR3 residue 91–96, and for DPL16 CDR3 position 92–95 and 95b). The scFv structure was displayed using the program PyMol, based on the protein data base (Brookhaven Protein Data Bank) file 1igm. The residue numbers were defined as previously published in reference 23 and 63. (B) Library cloning strategy. A point mutation was introduced, converting residue S52 of VH to D, K, N or Y. Mutagenesis in the CDR3 regions was generated by PCR using partially degenerate primers. Genes are indicated as rectangles and CDRs as numbered boxes. The VH and VL segment were then assembled by PCR and cloned into the pHEN1 vector.[64] Primers used in the amplification and assembly are listed in **Table 1**.

on the EDA domain of fibronectin, which did not overlap with the one recognized by the clinical-stage F8 antibody and which stained tumor neo-vascular structures in frozen cancer sections. One of these antibodies, termed 2H7, was fused to F8 in scFv-scFv format[34,35] by means of two different linkers. The resulting fusion proteins, named chelating recombinant antibodies (CRAbs),[34,36] were produced in mammalian cells and characterized for their tumor homing properties using quantitative biodistribution analysis in tumor-bearing mice.

Results

Library design, construction and characterization. To generate a large, stable and highly diverse library of functional antibody fragments with similar physical properties, we cloned a synthetic antibody library in a phagemid vector. Sequence diversity was restricted to the complementarity-determining region (CDR) 3 loops of the variable heavy (VH) and light chain (VL) domains (**Fig. 1A**). The scFv format was selected because it provides better expression yields and superior phage display properties compared with the Fab format. The scFv antibody scaffold used for the library was based on the germline VH segment DP47 and either the Vκ segment DPK22 or the Vλ segment DPL16 (**Fig. 1B**), which represent 12, 25 and 16%, of the antibody repertoire in humans, respectively.[6] This approach offers a number of advantages, e.g., higher thermodynamic stability,[37] possible use of Protein A for antibody purification and detection.[4] Sequence randomization was limited to the CDR3 loops, which are known to contribute substantially to antigen recognition (**Fig. 1**). A completely randomized sequence of four, five, six or seven amino acid residues (followed by the conserved Phe-Asp-Tyr sequence) was appended by means of polymerase chain reaction (PCR) to the VH germline segment (**Table 1**), thus forming short CDR3 loops, whereas a partially randomized sequence of six amino acid residues was appended to the VL, forming CDR3 loops that contained at least one proline residue (**Table 1**). The flexible polypeptide $Gly_4SerGly_4SerGly_4$,[38] was used to link the VH chain to the VL chain in the scFv fragments. Additionally, residue 52 in the CDR2 loop of the VH domain was allowed to be either two charged amino acids (Asp, Lys in PHILO1 sub-library) or two hydrogen bond-prone amino acids (Asn, Tyr in PHILO2 sub-library). The sub-libraries could be used together by mixing the corresponding phage preparations, resulting in the PHILO library, which contained 3.1 x 10^9 different antibody clones.

To assess the quality of the PHILO library, a number of tests were performed. First, 96/96 randomly chosen colonies exhibited the presence of an insert of the correct size in PCR screening experiments (**Fig. 2A**). Furthermore, 26 randomly picked clones were shown to contain the correct sequence and randomized residues at the desired positions (**Sup. Table 1**). A dot blot analysis, based on the detection of the peptidic myc tag at the C-terminus of the recombinant antibodies, confirmed that more than 85% of library clones could be expressed as soluble scFv fragments in the bacterial supernatant (**Fig. 2B**).

The PHILO library was screened against a panel of nine different antigens and in two competition selections, yielding several binders in all cases after two or three rounds of panning (**Table 2**). Except for the first four antigens reported in **Table 2** (GST, BCD, 11A12 and 7B89), which were used as standard selections, most of the different antibodies were further characterized by surface plasmon resonance analysis for K_D determination. Some clones specific for fibronectin domain, filtrin, nephrin and BstII were also used as immunohistochemical tools for antigen detection in human tumor sections (data not shown). To assess the epitope variability that can be recognized by our newly designed antibody library, we attempted to isolate antibodies specific for two new epitopes of EDA and EDB

Table 1. Sequence of synthetic primers

S52Drev	GCG TAG TAT GTG CTA CCA CCA CTA CCG TCA ATA GCT GAG ACC CAC TCC AG
S52Krev	GCG TAG TAT GTG CTA CCA CCA CTA CCC TTA ATA GCT GAG ACC CAC TCC AG
S52Nrev	GCG TAG TAT GTG CTA CCA CCA CTA CCG TTA ATA GCT GAG ACC CAC TCC AG
S52Yrev	GCG TAG TAT GTG CTA CCA CCA CTA CCA TAA ATA GCT GAG ACC CAC TCC AG
S52fw	GGT AGT GGT GGT AGC ACA TAC TA
(a) LMB3long	CAG GAA ACA GCT ATG ACC ATG ATT AC
(b1) DP47CDR301rev	GTT CCC TGG CCC CAG TAG TCA AAM NNM NNM NNM NNT TTC GCA CAG TAA TAT ACG GCC
(b2) DP47CDR302rev	GTT CCC TGG CCC CAG TAG TCA AAM NNM NNM NNM NNM NNT TTC GCA CAG TAA TAT ACG GCC
(b3) DP47CDR303rev	GTT CCC TGG CCC CAG TAG TCA AAM NNM NNM NNM NNM NNM NNT TTC GCA CAG TAA TAT ACG GC
(b4) DP47CDR304rev	GTT CCC TGG CCC CAG TAG TCA AAM NNM NNM NNM NNM NNM NNM NNT TTC GCA CAG TAA TAT ACG GC
(c) DP47CDR3fw	TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC
(d1) DPK22CDR301rev	CAC CTT GGT CCC TTG GCC GAA CGT MNN CGG MNN MNN ACC MNN CTG CTG ACA GTA ATA CAC TGC
(d2) DPK22CDR302rev	CAC CTT GGT CCC TTG GCC GAA CGT MNN CGG MNN ACC MNN MNN CTG CTG ACA GTA ATA CAC TGC
(e1) DPL16CDR301rev	CTT GGT CCC TCC GCC GAA TAC CAC MNN MNN MNN MNN MNN GGG AGA GGA GTT ACA GTA ATA GTC
(e2) DPL16CDR302rev	CTT GGT CCC TCC GCC GAA TAC CAC MNN MNN MNN MNN GGG MNN AGA GGA GTT ACA GTA ATA GTC
(e3) DPL16CDR303rev	CTT GGT CCC TCC GCC GAA TAC CAC MNN MNN MNN GGG MNN MNN AGA GGA GTT ACA GTA ATA GTC
(e4) DPL16CDR304rev	CTT GGT CCC TCC GCC GAA TAC CAC MNN GGG MNN MNN MNN MNN AGA GGA GTT ACA GTA ATA GTC
(e5) DPL16CDR305rev	CTT GGT CCC TCC GCC GAA TAC CAC GGG MNN MNN MNN MNN MNN AGA GGA GTT ACA GTA ATA GTC
(f) DPK22FR4NotIrev	TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAC CTT GGT CCC TTG GCC GAA CG
(g) DPL16FR4NotIrev	GAG TCA TTC TCG ACT TGC GGC CGC GCC TAG GAC GGT CAG CTT GGT CCC TCC GCC GAA
(i) fdseqlong	GAC GTT AGT AAA TGA ATT TTC TGT ATG AGG
DP74CDR1rev	CTG GAG CCT GGC GGA CCC AGC TCA TMN NMN NMN NGC TAA AGG TGA ATC CAG AGG CTG C
DP74CDR1fw	GAG CTG GGT CCG CCA GGC TCC
DPL16 CDR1rev	TCC TGG CTT CTG CTG GTA CCA GCT TGC MNN MNN MNN TCT GAG GCT GTC TCC TTG
DPL16 CDR1fw	TGG TAC CAG CAG AAG CCA GGA
CRAb1	CTA GCT AGC GTC GAC CAT GGG CTG GAG CCT GAT
CRAb2	AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TTT GAT TTC CAC CTT GGT CCC TTG
CRAb3	ACC AGA CGC TTT TCC GTC TGA GCC GGA GGA AGA ACC TTT GAT TTC CAC CTT GGT CCC TTG
CRAb4	GGT TCA GGC GGA GGT GGC TCT GAG GTG CAG CTG TTG GAG TCT G
CRAb5	CTC AGA CGG AAA AGC GTC TGG TGG CTC CGC GTC AGG CGG TGA GGT GCA GCT GTT GGA GTC TG
CRAb6	TCG ATA AGC TTT CAT TAT GCG GCC CCA TTC AGA TCC TCT TCT GAG ATG AGT TTT TGT TCT

M, A/C; N, A/C/G/T according to the IUPAC nomenclature.

of human fibronectin that did not compete with our previously described F8 antibody and L19 antibody, respectively.[15-17] In the case of anti-EDB antibodies we selected three different clones from the PHILO library and one from the ETH2-GOLD library (data not shown). For the EDA domain, we isolated one antibody specific for a new epitope from only the PHILO library.

Anti-EDA binders. A recombinant fibronectin fragment, constituted by the three type-III domains 11, EDA and 12 (hereafter termed "11-A-12"[15]) was used in panning experiments with both the ETH2-GOLD library[11] and the PHILO library, using the F8 antibody as competitor during selections to drive the isolation of binders that recognize alternative non-overlapping epitopes. After two rounds of panning, 21/94 ELISA-positive clones were recovered from the PHILO library and 45/94 from the ETH2-GOLD library. Characterization of the mAbs in terms of (1) EDA recognition and (2) absence of competition with F8 indicated that only clone F10 from the PHILO library could bind to EDA without competing with F8 (**Fig. 3A–C and Table 3**). **Figure 3A and B** present the results of competition ELISA and BIAcore experiments with subsequent antibody injections, using the F10 antibody or B7 (a different high-affinity anti-EDA antibody isolated from the phage selections[15] that maps on the same epitope of the previously described F8) in the presence of F8. Only the F10 antibody could recognize EDA in the presence of F8, while the binding of B7 was completely abrogated. Furthermore, F8 in the small immunoprotein (SIP) format and F10 in scFv format were able to form a triple complex in the presence of 11A12, as assessed by size-exclusion chromatography (SEC) analysis (**Fig. 3C**). The measured K_D value for scFv(F10) was 290 nM, prompting the use of an affinity maturation procedure by means of mutagenesis-based technique previously described by our group[11,15] (see also Materials and Methods), yielding clone 2H7 with a Kd = 50 nM dissociation constant to the cognate antigen as measured by BIAcore (**Table 3**). The 2H7 antibody retained the ability of the

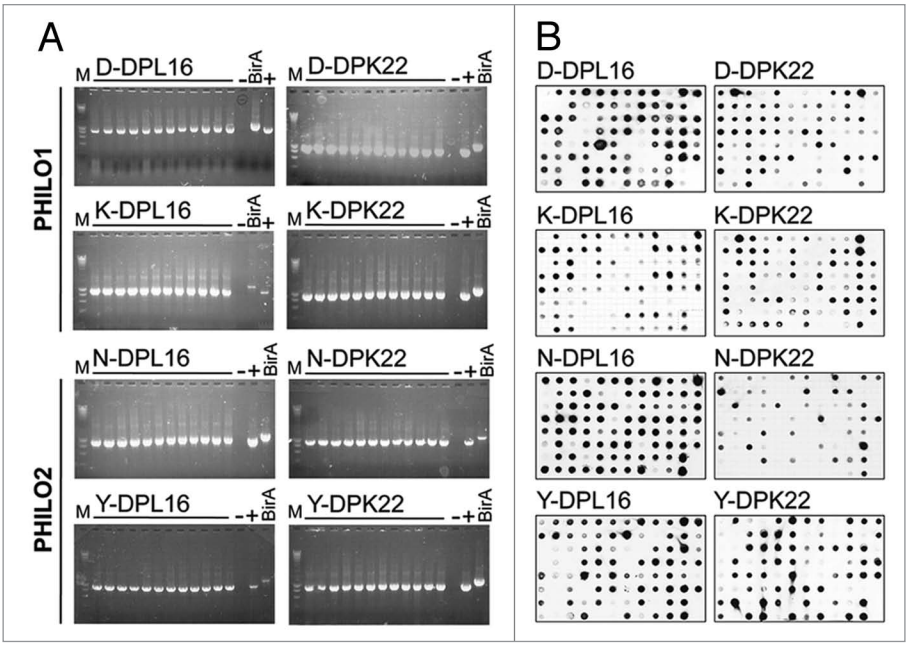

Figure 2. Characterization of the PHILO library. (A) PCR colony screening of 12 clones of each sub-library. As negative control a BirA insert (1,200 bp) of a pHEN1 vector was amplified and a scFv in pHEN1 was amplified as a positive control. All the tested clones showed an insert with the correct size of approximately 1,000 bp. (B) Dot blot analysis of 752 induced supernatants of individual library clones. The soluble scFv fragments were detected with the anti-myc-tag mAb 9E10. More than 90% of the clones express a detectable amount of soluble scFv fragment.

Table 2. Summary of test selections with PHILO and ETH2-GOLD libraries

	PHILO library				ETH2-GOLD library			
Antigen	**ROP**	**Pos**	**D.S.**	**Valid**	**ROP**	**Pos**	**D.S.**	**Valid**
GST	2	28/92	n.d.	-	2	25/94	n.d.	-
BCD Domain of murine tenascin-C	2	57/94	n.d.	-	2	50/94	n.d.	-
7B89 Domain of human fibronectin	2	43/94	n.d.	-	2	40/94	n.d.	-
11A12 Domain of human fibronectin	2	21/94	n.d.	-	2	46/94	n.d.	-
TIMP1	2	2/92	2	-	2	11/92	4	-
Human nephrin	2	8/92	4	IHC	2	5/92	3	IHC
Human filtrin	2	6/92	1	IHC	2	2/94	2	IHC
Mouse MMP2	3	3/92	1	200 nM	2	27/176	4	200 nM
Human BstII	2	17/92	4	248 nM	2	5/92	2	200 nM
EDB of human fibronectin competition with SIP(L19)*	2	3/94	3	45 nM	2	1/94	1	50 nM
EDA of human fibronectin competition with SIP(F8)**	2	7/94	1	290 nM	2	0/94	-	

ROP, rounds of panning. Pos, positive clone in ELISA screening (at least 20 times the value of the negative control). D.S., different amino acid sequence in VH-CDR3 and VL-CDR3 found in positive clones. Valid, validation of different clones performed either by immunohistochemistry (IHC, where at least one clone stained specific structures), or by BIAcore K_D determination (only value of the best clone is reported). *For a review see reference 65. **For a review see reference 15.

parental antibody to recognize EDA in the presence of F8 (data not shown).

Production and characterization of chelating recombinant recombinant anti-EDA antibodies (CRAbs). To generate tandem scFv-scFv molecules that would work as CRAbs specific to the EDA domain of fibronectin, we sequentially fused scFv(F8) and scFv(2H7) using two different flexible polypeptides of 10 and 18 amino acids as linkers joining the two antibodies. The resulting bispecific antibodies were termed CRAb(F8-10aa-2H7) and CRAb(F8-18aa-2H7) (**Fig. 4B**). SEC showed that both anti-EDA CRAbs were able to elute as a main peak at the same retention volume of scFv(F8)-5amino acid linker[15] (**Fig. 4B**). To test the tumor targeting performance of the new bispecific antibodies, purified preparations of CRAb(F8-10aa-2H7) and CRAb(F8-18aa-2H7) were radiolabeled with ^{125}I and injected intravenously (i.v.) in 129SvEv

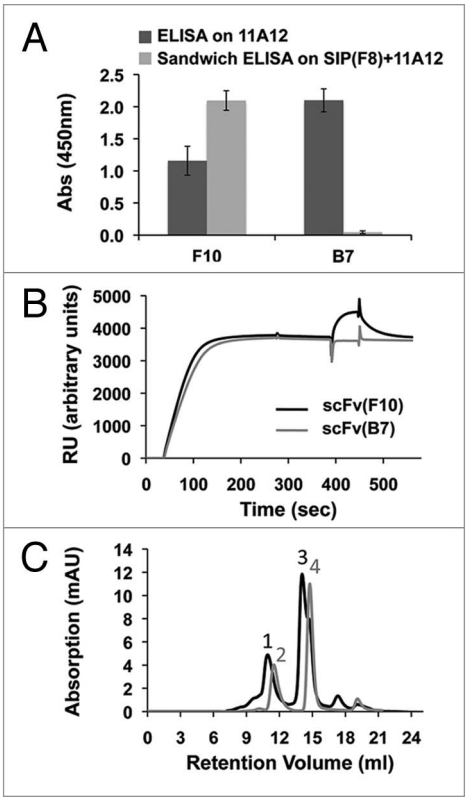

Figure 3. Experimental evidence of non-overlapping epitopes on EDA, recognized by the scFv(F8) and scFv(2H7) antibodies. (A) ELISA and sandwich ELISA results are depicted in dark grey and light grey column, respectively, using microtiter plates coated with the recombinant 11A12 fragment of fibronectin (ELISA) or the F8 antibody (sandwich ELISA). (B) BIAcore experiments were performed on 11A12-coated CM5 chips. The F8 antibody in the small immunoprotein (SIP) format[15] was injected at saturation, followed by injection of either scFv(F10) (black line) or the negative control scFv(B7) antibody (light grey). The EDA epitope recognized by scFv(B7) is identical to the one recognized by the F8 antibody. (C) Triple complex formation of SIP(F8), 11A12 and scFv(F10) in size exclusion chromatography on Superdex 200. Peak 1 corresponds to the triple complex made by SIP(F8) + 11A12 + scFv(F10); peak 2 corresponds to SIP(F8) + 11A12; peak 3 corresponds to scFv(F10) + 11A12; peak 4 corresponds to 11A12 antigen alone.

uptake of scFv(2H7)-diabody was lower compared with that of scFv(F8)-diabody, this antibody also exhibited a favorable tumor to organ ratio profile (**Fig. 4A**). Both anti-EDA CRAbs displayed a preferential accumulation at the tumor site with comparable levels in the neoplastic masses (**Fig. 4**). Twenty-four hours after i.v. injection, scFv(F8) and CRAb(F8-18aa-2H7) exhibited comparable tumor to blood ratios of 10.2 and 8.8, respectively. Furthermore, CRAb(F8-18aa-2H7) revealed a long residence time of the antibody fragment at the tumor site, with low background in healthy organs.

Discussion

We have reported the design, construction and characterization of the new synthetic human antibody library (i.e., the PHILO library), containing more than three billion different antibody clones. The previously generated human antibody library ETH2-GOLD[11] has been used for isolation of antibodies against more than 100 different antigens. The PHILO synthetic antibody library was generated to expand the structural features and epitope variability that can be recognized by our antibody fragments. The new library design capitalizes on the experience gained with the ETH2-GOLD library, but contains point mutations at position 52 of the VH CDR2 loop that help bias antibody selection towards certain protein epitopes. The PHILO library allowed the isolation of human antibodies specific to a novel epitope in the EDA domain of fibronectin, which we had previously not been able to obtain with existing libraries, and it has now allowed the construction of tumor-targeting CRAbs.

Among the various tumor-associated extra-cellular-matrix (ECM) components, oncofetal fibronectin isoforms are often recognized as ideal targets for antibody-based tumor targeting applications due to their high stability, ready accessibility and abundance.[39] The EDA and EDB domains of fibronectin are usually absent in both plasma and tissue fibronectin of adults, but they can be inserted into the fibronectin molecule during active tissue remodeling events, such as angiogenesis, wound healing and embryogenesis.[40,41] The anti-EDB L19 antibody[16-18] and the anti-EDA F8 antibody have been extensively studied as delivery vehicles in pre-clinical models of cancer and chronic inflammation.[41-45] The L19 antibody fused to the pro-inflammatory cytokines IL-2 or TNF or labeled with the β-emitting

Table 3. Sequences of the scFv CDRs specific to the extra-domain A of fibronectin

scFv	VH chain (DP-47)			VL chain		
	31–33[a]	52	95–100[a]	31–32[a]	91–96[a]	Germline
scFv(F10)	S Y A	N	YIRTKD	S Y Y	ITGYPV	DPL-16
scFv(2H7)	**K F K**	N	YIRTKD	**S Y Y**	ITGYPV	DPL-16
scFv(F8)	L F T	S	STHLYL	M P F	MRGRPP	DPK-22
Linkers						
CRAb (10 aa linker)	GGG GSG GGG S					
CRAb (18 aa linker)	GSS SGS DGK ASG GSA SGG					

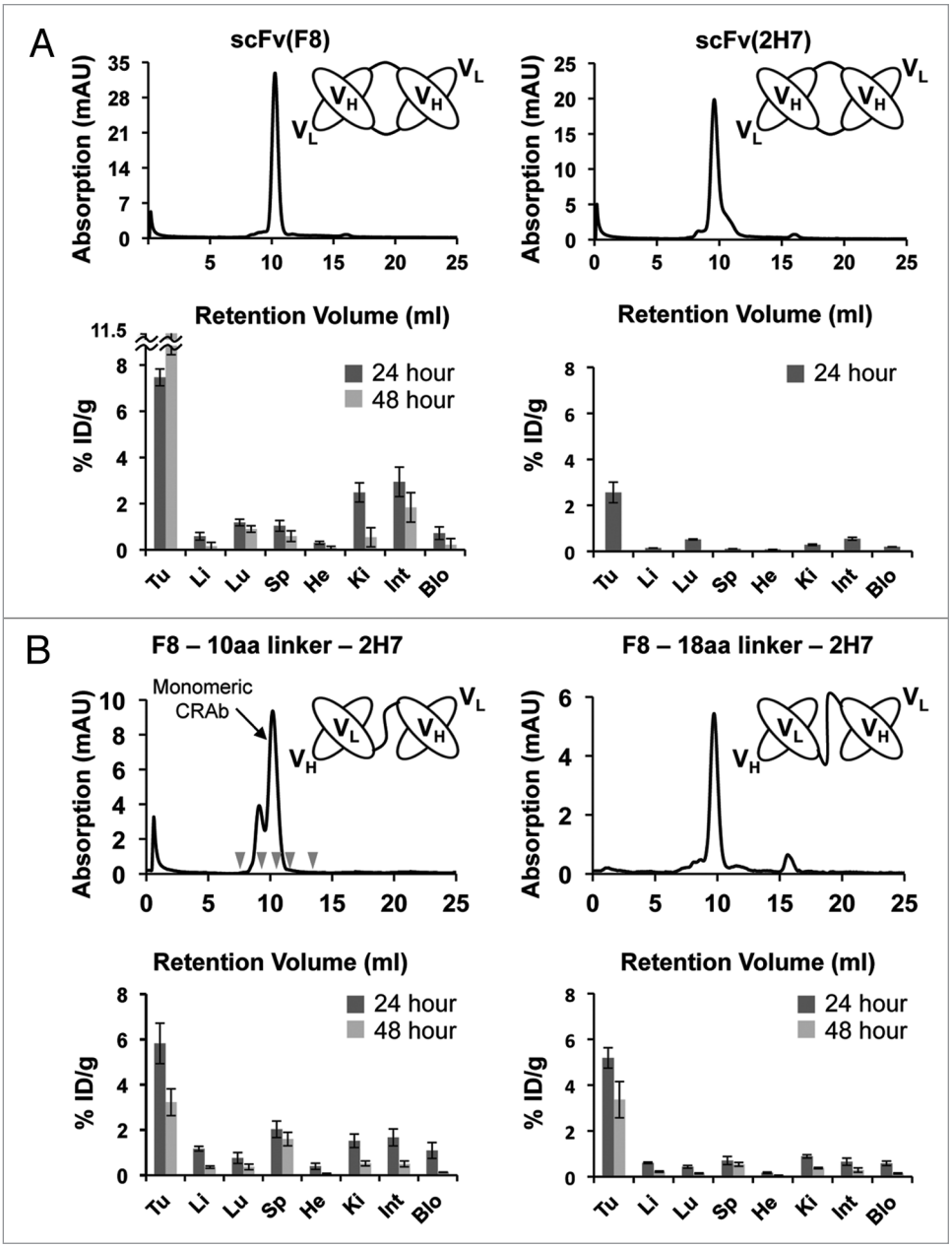

Figure 4. Biodistribution studies in tumor bearing mice of scFv(F8) and scFv(A9) in diabody format (A) and CRAbs (B). Both parts report the schematic representation of antibody structure, the size exclusion analysis of antibody fragments on Superdex75 (Amersham, GE) (all antibodies have a main elution peak around 10 mL, which corresponds to the expected size of 52–54 kDa) and the biodistribution analysis displayed as percent injected dose per gram (%ID/g) in tumor (Tu) and in measured mice organs: liver (Li), lung (Lu), spleen (Sp), heart (He), kidney (Ki), intestine (Int), blood (Blo). Radioactivity in tumor and organs have been counted at 24 (dark grey) and 48 (light grey) hours post intravenous injection of radiolabeled antibodies. In (B), in the SEC profile of F8-10aa linker-2H7, the peak corresponding to the monomeric CRAb is marked. Small grey arrows indicate the retention volumes of the standard proteins (from left to right: blue dextran, bovine serum albumin, ovalbumin, scFv(F8), ribonuclease A).

^{131}I radionuclide, is currently being evaluated in Phase 2 clinical trials.[46-48]

In vivo biodistribution experiments have confirmed that both anti-EDA CRAb(F8-10aa-2H7) and CRAb(F8-18aa-2H7) antibodies were able to selectively accumulate at the tumor site. Subtle variations in linker length and amino acid composition have a small but measureable influence on the %ID/g values measured in tumors and in normal organs (**Fig. 4C**).

Bispecific antibodies have been used extensively in biomedical research, particularly with the aim of bridging an effector cell (i.e., cytotoxic T lymphocyte by CD3 co-receptor engagement) with a tumor cell by means of tumor-associated antigens such as CD19,[49,50] epithelial cell adhesion molecule (EpCAM),[51] carcinoembryonic antigen (CEA),[52,53] human epidermal growth factor receptor 2 (HER2)/*neu*[54,55] or epidermal growth factor receptor (EGFR).[56,57] Multi-specific antibodies, such as scFv-scFv tandem antibodies, can also be used as modular building blocks for the development of biopharmaceuticals. Multi-specific immunotoxins capable of simultaneous binding to both EpCAM and HER2*neu* exhibited superior tumor cell killing properties and decreased toxicity in a mouse model of cancer.[58] Even though our targeting results obtained with the CRAb(F8-18aa-2H7) are encouraging, further work is needed to evaluate whether CRAbs may display superior targeting performance when compared to homobivalent antibodies. In our biodistribution studies, anti-EDA CRAbs and diabodies exhibited comparable tumor targeting performance (**Fig. 4C**).

In summary, we believe that our new synthetic antibody PHILO library represents a useful source for the isolation of novel mAb fragments specific for virtually any type of antigen. Furthermore, scFv-scFv tandem antibodies may be useful modular building blocks of proven tumor targeting performance for the development of improved anti-cancer biopharmaceuticals.

Materials and Methods

Library construction and cloning. Two clones from the ETH2-Gold library[11] were used as template for PCR amplification of DP47, DPK22 and DPL16. Antibody residues are numbered according to Tomlinson[22] and Cox.[24] First, the point mutation at

position 52 of heavy chain was introduced by PCR using primers LMB3long, S52Drev, S52Krev, S52Nrev, S52Yrev, S52fw, i (**Table 1**; all primers used for the construction of the library were purchased from Operon Biotechnologies). The $G_4SG_4SG_4$ linker between heavy and light chain was not changed compared to the ETH2-GOLD library. Sequence variability in both heavy and light chain was focused in CDR3 loops and introduced by PCR using partially degenerated primers (**Table 1**), essentially as described in Silacci et al.[11] Briefly, DP47-based VH domains were randomly mutated from residue 95 to 100; this CDR3 loop was designed to be 4–7 amino acids long. DPK22-based VL domains were randomized between residues 91–96, with a fixed glycine either at position 92 or 93. Furthermore, at least one of the CDR3 loop residues was requested to be a proline.

Randomized CDR3 loops were appended to germline segments (from framework1 to framework3) by PCR using primer pairs a/b1-4 for DP47 heavy chain, c/d1-2 for DPK-22 light chain, and c/e1-5 for DPL-16 light chain (**Table 1 and Fig. 1**). After gel-purification, heavy and light chain segments were assembled by PCR and further amplified using primers a/f for DPK-22 or a/g for DPL-16. The resulting scFv genes were doubly digested with *Nco*I/*Not*I and cloned into the *Nco*I/*Not*I-digested expression vector pHEN1.[4] The ligation mixture was purified and electroporated into fresh electrocompetent TG-1 cells. Electrocompetent TG-1 cells were prepared by washing the cells twice with 1 mM HEPES/5% glycerol and twice with 10% glycerol in water. Finally, cells were resuspended in 10% glycerol to a density of approximately 2 x 10^{11} cells. Electroporated cells were spread on 2TY-agar plates (2xTY: 16 g/L bacto-tryptone, 10 g/L bacto-yeast extract, 5 g/L NaCl pH 7.4–15 g/L agar-100 μg/mL ampicillin, 1% glucose) and incubated at 30°C overnight. On the next day, cells were rescued (with 2TY-10% glycerol), used for phage production according to standard protocol[21] and stored as glycerol stocks. The sublibraries were electroporated in eight different days, yielding a total library size of 3.1 x 10^9 antibody clones (**Table 2**).

Library characterization: PCR screening, sequencing of antibody clones, dot blot. A total of 96 clones were tested by PCR screening with REDTaq Ready Mix PCR reaction mix (Sigma Aldrich GmBH, cat. num. R2523-100RXN) using primers **a/i** (annealing about 100 bp upstream/downstream of the scFv gene, **Table 1**). Antibodies were sequenced using Big Dye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, cat. num. 4337455) on an ABI PRISM 3130 Genetic analyzer. Termination reactions were performed on PCR screening products using primers **a** or **i** (**Table 1**). Dot Blot analysis was performed as described in Villa 2008,[15] individual colonies from the plated library were inoculated in 160 μL 2xTY-100 μg/mL ampicillin-0.1% glucose in Nunclon™ Surface 96-well plates (Nunc, cat. num. 163320). The plates were incubated 3 h at 37°C in a shaker incubator. Expression was induced by addition of 1 mM IPTG and cultures were grown overnight at 30°C. ScFv-containing supernatants were blotted onto Protran B85 0.45 μm nitrocellulose membrane (Whatman, cat. num. 10401197) using the ELIFA system (Pierce, cat. num. 77000), which allowed blotting by vacuum in a 96-well format, leading to spots of uniform and reproducible size. ScFv was detected with monoclonal anti-myc tag murine antibody 9E10,[59] followed by anti-mouse IgG horseradish peroxidase conjugate (Sigma Aldrich, A2554). Peroxidase activity was detected using the ECL plus western blotting detection system (Amersham Biosciences, GE Healthcare, cat. num. RPN2132) on Amersham Hyperfilm ECL (Amersham GE, cat. num. 28-9068-35).

Antibody selection. All selections were performed as described in Silacci 2005,[11] using recombinant antigen with >90% purity as assessed by SDS-PAGE and SEC. Immunotubes (Nunc, cat. num. 470319) were coated with antigen at 10^{-6} M concentration in phosphate buffered saline (PBS) overnight at room temperature (RT) and blocked for 2 h at RT with 2% w/v skimmed milk in PBS (MPBS). After rinsing with PBS, $>10^{12}$ phage particles were added to the antigen-coated immunotube in the presence of 2% MPBS, incubated for 30 min at room temperature with shaking (100 rpm), followed by 1.5 h incubation at RT without agitation. In competitive selections, phage particles were added to the antigen-coated immunotube in the presence of 2% MPBS and 10^{-5} M of competing antibody (SIP(L19) for anti-EDB selection and SIP(F8) for anti-EDA selection). Unbound phage were washed with PBS Tween 0.1% (10 or 20 times) and PBS (10 or 20 times), while bound phage were eluted with 100 mM triethylamine (TEA). Eluted phage were then used for infection of exponentially growing *E. coli* TG1. After 2 rounds of panning, ELISA screening was performed on 92–94 individual colonies as previously described (Silacci 2005).

ELISA screening and sandwich ELISA. Bacterial supernatants containing scFv fragments were screened for antigen binding ability by ELISA as described in reference 21. Individual colonies were inoculated in 180 μL 2x TY, 100 μg/mL ampicillin (Applichem, cat. num. A0839, 0025), 0.1% glucose (Sigma Aldrich) in Nunclon™ Surface 96-well plates (Nunc, cat. num. 163320). The plates were incubated 3 h at 37°C in a shaker incubator. The cells were then induced with isopropyl-thio-galactopyranoside (IPTG; Applichem, cat. num. A1008, 0050) at a final concentration of 1 mM and grown overnight at 30°C. The bacterial supernatants assayed were tested in ELISA experiments as described in reference 60 using the anti-myc tag 9E10 mAb (Sigma, cat. num. M4439) and anti-mouse horseradish peroxidase (HRP) immunoglobulins (Sigma Aldrich, cat. num. A2554) as secondary reagents. For the colorimetric reaction, 100 μL BM-Blue POD substrate (Roche, cat. num. 11484281001) followed by 50 μL 1 M H_2SO_4 were added to each well. The absorbance was measured using the microtiter plate reader (VersaMax, Molecular Devices) at wavelengths 450 nm and 650 nm. Sandwich ELISA was performed by coating 100 μL (100 μg/mL) anti-EDA antibody SIP(F8)[15] directly on Maxisorp (NUNC, cat. num. 439454), followed by addition of 100 μl 11A12 triple domain of fibronectin[15] at a concentration equal to 10^{-5} M. Subsequently, scFv(F10) or scFv(B7),[15] were added at a concentration of 50 μg/mL. The anti-myc tag 9E10 antibody and anti-mouse HRP were used for ELISA development.

BIAcore. Surface plasmon resonance analysis was carried out with BIAcore 3000 system (BIAcore, GE Healthcare) using an 11A12 triple domain of human fibronectin-coated CM5

chip (BIAcore, cat. num. BR-1000-14) at 10 μL/min flow rate. Twenty microliters of SIP(F8) were injected at a 0.1 mg/mL concentration to achieve binding site saturation of the antigen-coated chip. Subsequently, 20 μL of scFv(F10) or scFv(B7) were injected at the same concentration of 0.1 mg/mL. Regeneration of the chip was performed by injecting 20 μL of HCl 10 mM.

Size-exclusion chromatography. Purified antibody fragments and antigen mixtures were analyzed by SEC on a Superdex 75 10/300 GL column on an ÄKTA FPLC (GE Healthcare, Amersham Biosciences). Before the analysis, column elution volumes were calibrated using different commercially-available protein standards for gel filtration calibration, such as ribonuclease A as 13.7 kDa reference (elution volume (V_e) = 13.346 mL), ovalbumin as 43 kDa reference (V_e = 10.095 mL), BSA as 67 kDa reference (V_e = 9.232 mL) and blue dextran for void volume (V_0) calculation (V_0 = 7.72 mL) (Amersham Biosciences, cat. num. 28-4038-42). The calibration graph was obtained by plotting Kav (defined as V_e - V_0/V_t - V_0, given a total column volume (V_t) = 25 mL), versus molecular weights (MW) of the standards. The equation of the trend line approximating the points [MW; Kav] is y = -5E-06x + 0.3702 (R^2 = 0.93). Before FPLC analysis, protein mixtures were incubated at room temperature for 20 min to allow binding equilibrium to be reached.

Affinity maturation of scFv(F10). The affinity maturation of scFv(F10) was performed according to a previously described strategy.[15,19,25] An affinity maturation library was cloned by introducing sequence variability in the CDR1 loops of both heavy (at position 31–33) and light chains (at position 31a, 31b, 32). Partially degenerate primers used for library construction are listed in **Table 1**. First, three fragments were obtained by PCR on the parental clone scFv(F10) as template, using primer pairs **a/DP74CDR1rev**, **DP74CDR1fw/DPL16CDR1rev** and **DPL16CDR1fw/i** (**Table 1**). After gel-purification, these three segments were assembled by PCR and further amplified using primers **a/i** (**Table 1**). The VH-VL combinations were doubly digested with *Nco*I/*Not*I and cloned into the *Nco*I/*Not*I-digested expression vector pHEN1. ScFv(2H7) was isolated by selection on Immunotube, followed by an ELISA screening procedure as reported above.

CRAbs cloning. CRAb genes were constructed by PCR amplification of the scFv(F8)[15] and scFv(2H7), followed by PCR assembly of the two obtained DNA fragments. ScFv(F8) was amplified using the primers **CRAb1/CRAb2** for F8-10aa-2H7 and **CRAb1/CRAb3** for CRAb(F8-18aa-2H7).[34] The myc-tagged scFv(2H7) gene was amplified using primer **CRAb4/CRAb6** (annealing on C-terminal myc-tag) for F8-10aa-2H7 and **CRAb5/CRAb6** for CRAb(F8-18aa-2H7). The obtained scFv genes were assembled by PCR, double digested with *Nhe*I/*Hind*III and ligated in double digested pCDNA3.1.

Expression and purification of CRAbs and scFv(F8)-5aa linker in CHO-S cells. Recombinant antibody fragments were expressed in suspension-adapted CHO-S cells by means of transient gene expression technique.[61] Antibody fragments were purified from culture supernatants by affinity chromatography, using protein A Sepharose (GE-Healthcare, cat. num. 17-5280-04), followed by elution either in glycine 100 mM pH 3 or in 100 mM TEA, depending on the pI of the eluted protein. Purified antibody fragments were analyzed by SEC on a Superdex 75 HR 10/30 column (GE-Healthcare, cat. num. 17-5174-01). Protein concentration was estimated from optical density measurements at 280 nm and SDS-PAGE analysis.

Tumor cell line and mouse model. F9 murine teratocarcinoma cells (CRL-1720, ATCC) were cultured according to ATCC guidelines. F9 tumor-bearing mice were obtained by injecting s.c. 10^6 F9 teratocarcinoma cells in 10 week-old female 129SvE mice (provided by Taconic, Ry, Denmark), as described in reference 15.

Biodistribution studies. The in vivo targeting performance of anti-EDA antibody fragments was evaluated by quantitative biodistribution analysis as previously described by Tarli et al.[18] Briefly, antibodies were first loaded on Superdex 75, and the peaks corresponding to the desired molecular weight (according to the equation reported above) were collected. Purified antibody preparations were then radiolabeled with ^{125}I using the chloramineT method[62] and injected into the tail vein of immunocompetent 129SvEv mice bearing s.c. implanted F9 murine teratocarcinoma (8–10 μg per mouse). Mice were sacrificed 24 and 48 h after injection. Organs were weighed and radioactivity was counted with a PackardCobra gamma counter. Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g). Data were corrected for tumor growth correction.

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Note

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