

Multivalent scFv Display of Phagemid Repertoires for the Selection of Carbohydrate-specific Antibodies and its Application to the Thomsen–Friedenreich Antigen

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The Thomsen–Friedenreich disaccharide (TF) is a promising target antigen for tumor immunotherapy, since it is almost exclusively expressed in carcinoma tissues. The TF-specific antibodies generated so far are IgMs of mouse origin with limited therapeutic potential. Phage-displayed scFv repertoires are an established source for recombinant antibodies; however, we were unable to identify scFvs binding to TF when applying libraries in the standard monovalent display format of phagemid systems. Here, we report on the successful selection of TF-specific antibody fragments using a multivalent scFv phagemid library format based on shortened linkers (one amino acid residue). The libraries were constructed from mice immunized with asialoglycophorin and selected using TF displayed on two different carrier molecules in combination with the proteolytically cleavable helper phage KM13. All isolated clones encoded the same framework genes and the same complementarity-determining regions. After affinity maturation only scFv with the founder sequence were selected from secondary repertoires. This indicates a very narrow sequence window for TF-specific antibodies. Investigating other linker-length formats revealed a clear inverse correlation between linker length and binding activity both as soluble proteins and displayed on phages. The highest affinity was obtained with the tetrameric format. The selected scFv was specific for TF on various carrier molecules and tumor cells and performed well in ELISA and immunohistochemistry. We postulate that scFv phagemid library formats with short linkers (i.e. multimeric scFvs) may, in general, be advantageous in selections for the generation of scFvs against carbohydrate epitopes or other epitopes associated with low intrinsic affinity per binding site), and expect that they will be superior in applications for diagnosis or therapy.

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Keywords: phage display; antibody engineering; multivalent scFv; tumor targeting; Thomsen–Friedenreich antigen

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Abbreviations used: aGP, asialoglycophorin; amp, ampicillin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IMAC, immobilised metal affinity chromatography; kana, kanamycin; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; scFv, single chain fragment variable; V_H and V_L, variable region of the heavy chain and light chain, respectively; TF, Thomsen–Friedenreich; aa, amino acid.

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Introduction

Most antibodies developed against tumor-associated or tumor-specific antigens target proteins;¹ however, the excellent tumor specificity of some glycans such as Tn, sialyl-Tn, TF, GloboH, LeX, sialyl-LeX, and LeY² should inspire the search for anti-carbohydrate antibodies. The majority of anti-carbohydrate antibodies generated so far have been of the IgM class, and are therefore not suited for *in vivo* diagnostics or therapy. As an alternative, recombinant antibodies are a promising source of antibodies for immunotherapy.

Among tumor-specific carbohydrate antigens, the Thomsen–Friedenreich antigen (TF, Gal β 1-3GalNAc α 1-O-Ser/Thr, also known as core-1 structure or TF α), seems particularly promising as a therapeutic target.^{3–5} TF is an oncofetal antigen, which is cryptic in healthy adults⁶ but becomes exposed as a *de novo* antigen on tumor cells due to incomplete O-glycosylation of mucins and related glycoproteins.^{7,8} TF has been found on various carcinomas, of which breast and gastrointestinal carcinomas as well as liver metastasis from colon cancer seem to be promising targets for a TF-based immunotherapeutic strategy.^{4,6,9–13} TF can be experimentally demasked on glycoproteins such as glycophorin by treatment with neuraminidase,^{14,15} and has been successfully used in vaccination trials with asialoglycophorin.⁵

All monoclonal antibodies with specificity for TF and applicability in immunohistochemistry are of the IgM class: BM22,¹⁶ HH8,¹⁷ RS1-114,¹⁸ A78-G/A7,¹⁴ and HB-T1 (DakoCytomation, Hamburg, Germany). Of these, A78-G/A7 was instrumental in the assignment of TF as a CD marker (CD176)¹⁹ and shows the best combination from tumor-specificity and affinity. The potential of TF-specific antibodies was shown in a tumor prevention model, where the IgM A78-G/A7 could inhibit the formation of liver metastases.¹³ For a broader application in immunotherapy, we are interested in recombinant antibodies against TF.

One of the most successful strategies for generating recombinant antibodies is by phage display.²⁰ Phage display of antibody fragments in a monovalent display format has been applied in most cases, but this may not be optimal when dealing with carbohydrate antigens, since efficient binding of carbohydrate-specific antibodies seems to depend on multivalency.^{20,21} The simplest multivalent format of recombinant antibodies is based merely on the scFv. Due to steric hindrance, formation of dimeric, trimeric and tetrameric complexes can be observed when the linker between the V_H and V_L is shortened.^{22,23} These small multivalent antibodies are generally assumed to exhibit superior biokinetics compared to traditional antibodies and monovalent scFv.

Considering these facts, we decided to investigate the potential of displaying multivalent scFv complexes on phage particles for the isolation of recombinant antibodies to carbohydrate antigens that form multivalent scFv complexes in solution. Here, we describe a novel strategy for cloning scFv repertoires in a format based on a short linker (one amino acid residue) between V_H and V_L, thereby achieving display of multimers using a phagemid vector. We have applied the KM13 helper phage,²⁴ which has an engineered trypsin cleavage site between domains 2 and 3 of the minor phage coat protein III. Trypsination of phages produced with this helper phage renders non-displaying phage particles unable to infect due to the cleavage of pIII encoded by KM13. Simultaneously, the myc-tag between the scFv and pIII encoded by the

phagemid will be cleaved. Trypsination was therefore used for elution leading to a successful infection of bacteria only by those phages carrying scFv prior to binding to the target molecule. Both effects taken together decrease the background by several orders of magnitude and enable an effective selection in a few rounds of panning only, thereby retaining diversity, reducing effects due to growth advantages, and avoiding selection bias toward dominating epitopes.^{25–28} Applying these strategies, we report on the first successful isolation of TF-specific multivalent recombinant antibodies.

Results

Generation of multivalent scFv phagemid libraries

Spleen cells from one Balb/c and one C3H/J mouse immunised with TF-carrying asialoglycophorin (aGP) were used for RNA extraction. A fraction of the splenocytes was cultivated in order to test the supernatants for antibody response in ELISA.²⁹ Only the culture supernatant from C3H/J splenocytes indicated the presence of TF-binding antibodies (data not shown). Spleen cells from one Balb/c mouse were cultivated for two days before RNA extraction. Half of these cells were cultivated in the presence of 2 μ g/ml of aGP for a potential specific stimulation.

Single-stranded cDNA covalently bound to magnetic beads was prepared from the RNA extracted from the four splenocyte preparations. cDNA-immobilisation on magnetic beads enables multiple PCR amplifications using the entire cDNA pool as template because the cDNA can be retrieved and thus ensures maximal diversity. The PCR amplification on the cDNA from the C3H/J mouse gave very limited amounts of variable gene products, whereas all three Balb/c preparations (“non-cultured”, “cultured”, and “cultured/stimulated”) resulted in several micrograms of PCR product. Single-chain Fv libraries in one amino acid residue linker formats were generated from the three preparations as described in Materials and Methods and illustrated in Figure 1. In parallel, three libraries were generated from the same material in the “classic” 18 residue linker format. All the libraries contained more than 5×10^6 individual transformations. PCR on single clones indicated insert ratios of 90–95% ($N=20$, respectively), and sequencing indicated good diversity of the libraries, since no identical clones were identified ($N=16$, respectively).

Selection of TF α -specific antibodies from immunized multivalent scFv phagemid libraries

The selection strategy was based on panning on aGP-coated immunotubes followed by biopanning with soluble TF α -PAA-biotin and streptavidin-conjugated magnetic beads in successive rounds of selection. The display of TF on different carriers

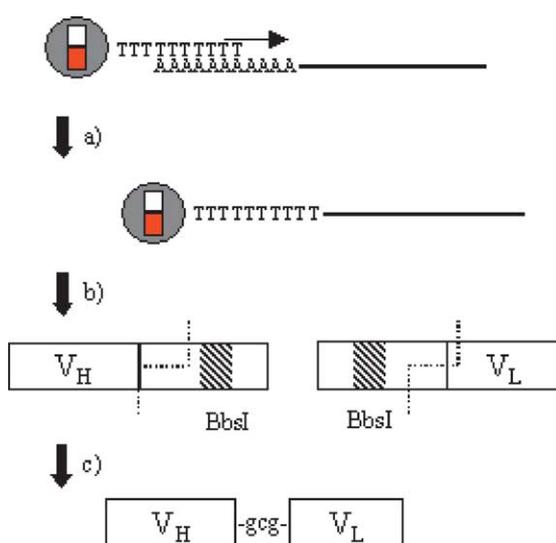


Figure 1. (a) Schematic presentation of library construction. The mRNA was captured on oligo(dT₂₅) magnetic beads, and single-stranded cDNA was synthesised using the beads as template. (b) The variable genes were amplified by PCR using all the generated cDNA as template in the reactions. (c) The variable genes were digested with BbsI, and ligated to form scFv genes, which were subsequently cloned into pHEN2.

directs the selection toward TF and minimises the selection of antibodies to the carriers. KM13 was employed as helper phage, and bound antibody-displaying phages were eluted by trypsin. After the second round of panning of the libraries, 10³ to 10⁴ clones were retrieved from each of the different libraries (Supplementary Material, Table 1). A total of 90 clones from each library were tested in phage ELISA in a primary screening against aGP, indicating binders in the selections of all the six libraries. However, a more stringent second screening, in which clones were assayed for binding both aGP and TF-PAA but not GP or BSA, identified only nine TF-specific clones and only from the libraries in the one amino acid residue format which were generated after culturing the splenocytes.

Sequencing of the nine clones binding both aGP and TF-PAA revealed the same complementarity-determining regions (CDRs) and V_H and V_L framework genes except for some variation in the primer-encoded regions.

In addition, several clones were selected that bound to aGP but not to either glycoprotein or TF-PAA. They were probably directed against peptide epitopes that are masked on fully glycosylated glycoprotein, an observation analogous to what we have seen and examined in detail in experiments to generate mouse monoclonal antibodies against TF (data not shown).

In search for additional TF-specific clones, we pooled the cultured and cultured/stimulated scFv(1aa) repertoires and performed a new first round panning against aGP, followed by two

rounds of biopanning against TF-PAA-biotin using 1 µg/ml in the second round and 100 ng/ml in the third round. Around 1500 clones were retrieved after the third round of selection. More than a third of the clones (322 out of 920) bound to aGP in the primary phage ELISA screening. Of those, 25 clones were identified as truly TF-specific binders in the second screening. Again, all 25 clones encoded the same V_H and V_L framework genes and the same CDRs with variations only in the primer encoding regions. A total of nine primer sequence combinations were identified, of which one sequence was found in 18 of the 34 clones, and therefore chosen for the further investigation.

Characterisation of different formats of the TF-specific scFv displayed on phage particles

The multivalent libraries constructed and employed herein were restricted to the scFv(1aa) format, which may not be the optimal linker with respect to expression, folding and stability of the scFv when displayed on phage particles. Therefore, the scFv sequence was cloned into pHEN2 in scFv formats covering all linker lengths from 0–9 and 18 residues. Displayed on phages, clones with linker lengths up to five residues performed better than those with longer linkers when tested for their binding activity in ELISA (Supplementary Material, Figure 1).

We have tested whether the displayed scFvs occur as multimers by combination of several full-length scFv-pIII-fusion proteins or as multimers consisting of a mixture of scFv-pIII-fusion proteins and non-fused scFv by Western blot analysis of purified phage particles.³⁰ No significant differences in the display level could be detected, and all constructs seemed to be associated with similar amounts of non-fused scFv (Supplementary Material, Figure 2).

Expression and purification of TF-specific scFv as soluble proteins

All scFv constructs scFv(0aa) to scFv(9aa) and scFv(18aa) were cloned into pET11a and expressed in Rosetta™. Crude TES fractions were prepared and tested as serial dilutions in ELISA against aGP (Figure 2). scFv(0aa) and scFv(1aa) showed the highest binding activity (measured as absorbance) in this assay. The amount of recombinant protein in the TES fractions was compared on a Western blot showing that the differences in activity could not be explained by differences in the amount of protein (Supplementary Material, Figure 3). These data correlate well with the data from the phage ELISA using cleared phage supernatants (Supplementary Material, Figure 1). The results were confirmed using various scFv formats purified by immobilised metal affinity chromatography (IMAC) from TES fractions obtained from two to four litre flask cultures. All preparations were more than 95% pure according to SDS-PAGE analysis.

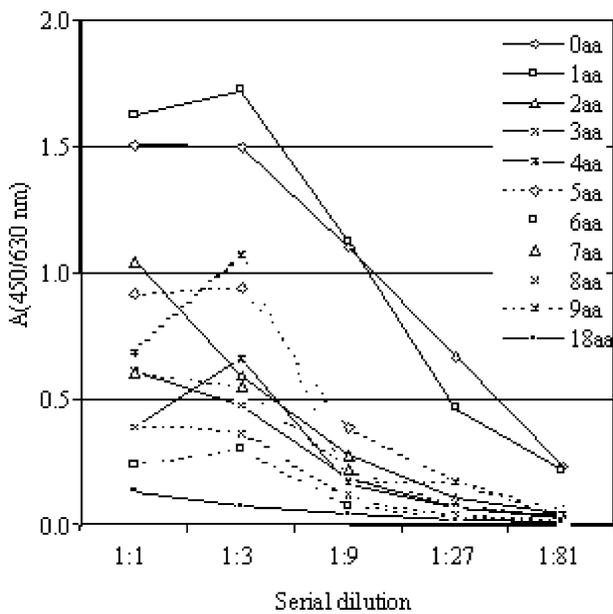


Figure 2. Analysis of soluble antibody formats expressed in bacteria. Crude extracts from bacteria expressing the different scFv constructs were analysed for activity in ELISA against aGP; $N=2$.

We tested the different scFv(1aa) clones with variations in the primer-encoded regions, but observed only minor differences in yield between the different clones, and no significant differences in ELISA activity. This indicates that the differences in the primer regions are not critical for binding activity or expression yield.

Size-exclusion chromatography

The multimerisation grade of the different scFv formats was analysed by size-exclusion chromatography on a G200 column. Examples of elution profiles are shown in Figure 3. The scFv(18aa) eluted with a peak at 56 kDa and a shoulder at 28 kDa, corresponding to a mixture of monomers and dimers with the majority in the dimeric fraction. The scFv(2aa) eluted at 56 kDa, corresponding to the formation of dimers, while the scFv(0aa) eluted at around 120 kDa, indicating the formation of larger multimeric, presumably tetrameric complexes. Gel-filtration of the scFv(1aa) gave a peak around 70 kDa, which is between the size of the dimers and that of assumed trimers. This indicated that the scFv(1aa) either forms a mixture of dimeric and trimeric complexes or that the trimers disintegrate to form dimers. The latter hypothesis was supported by the observation that the "dimer fraction" of the peak eluted as a dimer in a re-run, whereas the "trimer fraction" also eluted as a dimer (data not shown). We assume that the equilibrium between the dimeric and trimeric complexes of the scFv(1aa) was shifted toward the dimeric form on the column. However, the equilibrium was not affected by long-term incubation at

37 °C in dilute concentrations, where we have been unable to detect any differences in the M_r distribution. In contrast to the scFv(1aa), the tetrameric complexes of scFv(0aa) withstood the column conditions and eluted as a tetramer (data not shown). The scFv(3aa), scFv(4aa), and scFv(9aa) formed dimers (not shown).

Analysis of the fine-specificity of the scFv(1aa)

The fine-specificity of the purified scFv(1aa) was mapped in ELISA using a large array of more than 80 carbohydrate-PAA conjugates. aGP, which carries 16 TF groups,³¹ was included as positive control, and glycophorin, on which the corresponding TF epitopes are masked by sialic acids, served as

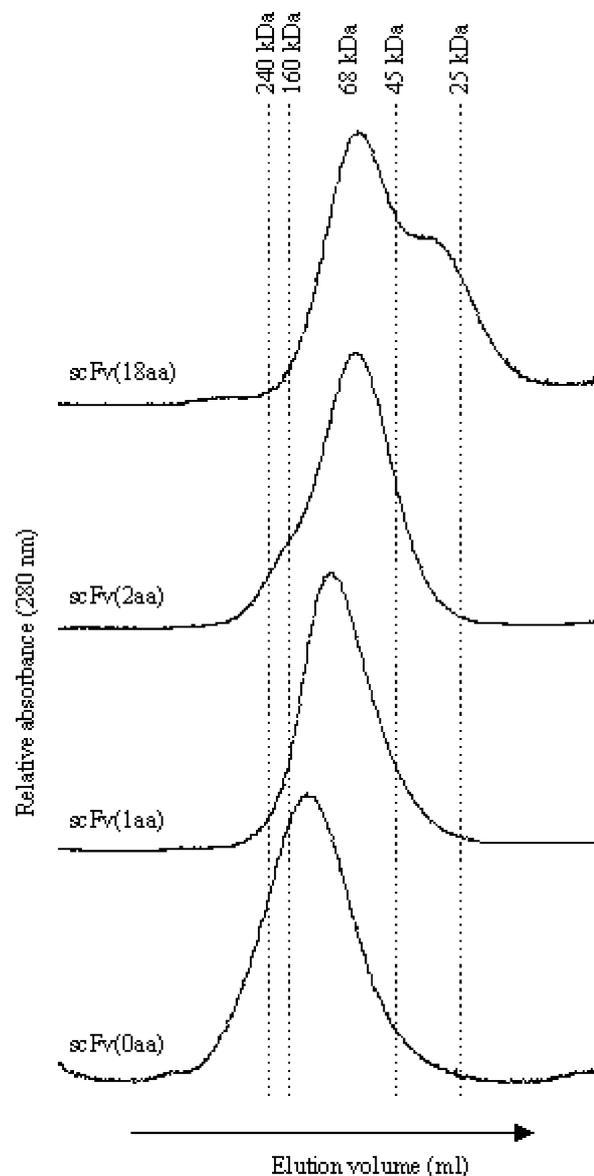


Figure 3. Size-exclusion chromatography of scFv constructs. IMAC-purified scFv(18aa), scFv(2aa), scFv(1aa) and scFv(0aa) constructs were analysed on a calibrated G200 column equilibrated in PBS (pH 7.0) at a flow rate of 0.1 ml/minute.

the corresponding negative control (Figure 4). The carbohydrate specificity was confirmed by the loss of binding after mild periodate oxidation of TF-determinants (data not shown). Strongest binding was detected against TF α (the natural protein-bound TF structure) displayed on aGP or conjugated to PAA *via* a flexible linker ($-\text{OCH}_2\text{CH}_2-\text{CH}_2\text{NH}-$). Reduced binding was also observed toward TF α linked to PAA with a more constrained linker ($-\text{OC}_6\text{H}_4\text{NH}-$). Minor binding was observed to core-2 (GlcNAc β 1-6[Gal β 1-3]GalNAc α), whereas the small signal with Gal α 1-4GlcNAc β was caused by a minor cross-reactivity of the tetra-His/HRP

antibody to this structure, and was not observed when the anti-myc antibody (9E10) was applied as secondary antibody. No other related or non-related carbohydrate structure was bound.

Real-time binding kinetics

The binding kinetics of the different constructs was evaluated by real-time surface plasmon resonance with a BIAcore instrument. We used experimental conditions of high density of aGP in order to allow multivalent binding of the constructs to TF. As an example, the sensorgram for the scFv(1aa) is shown

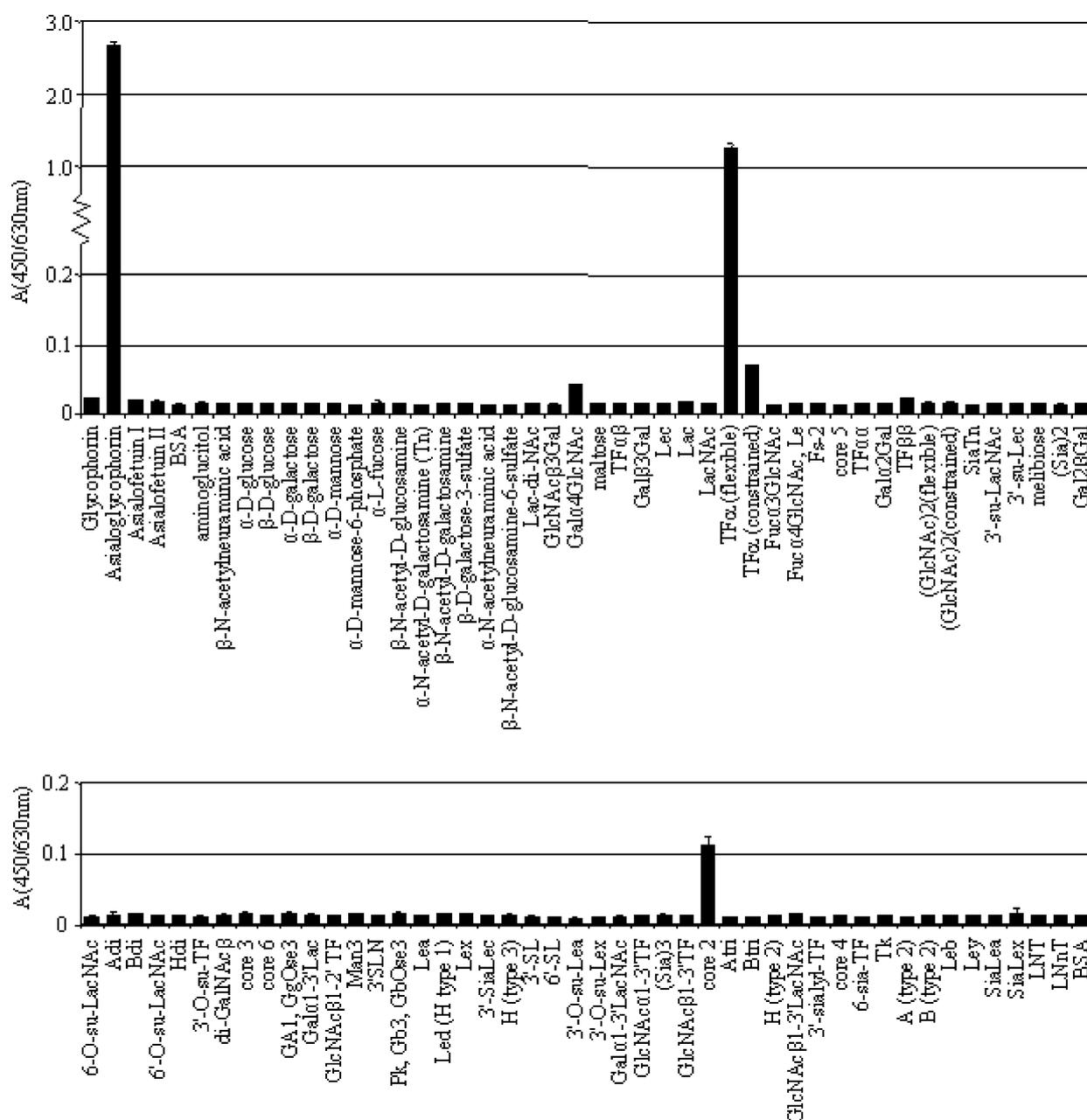


Figure 4. Specificity testing of scFv(1aa) with oligosaccharide-PAA conjugates in ELISA. The different oligosaccharide-PAA conjugates were coated at the solid phase, and probed for binding with scFv(1aa). Detection was performed with tetra-His/HRP; $N=2$. TF α , Gal β 1-3GalNAc α ; TF β , Gal β 1-3GalNAc α ; TF $\alpha\alpha$, Gal α 1-3GalNAc α ; TF $\alpha\beta$, Gal α 1-3GalNAc β .

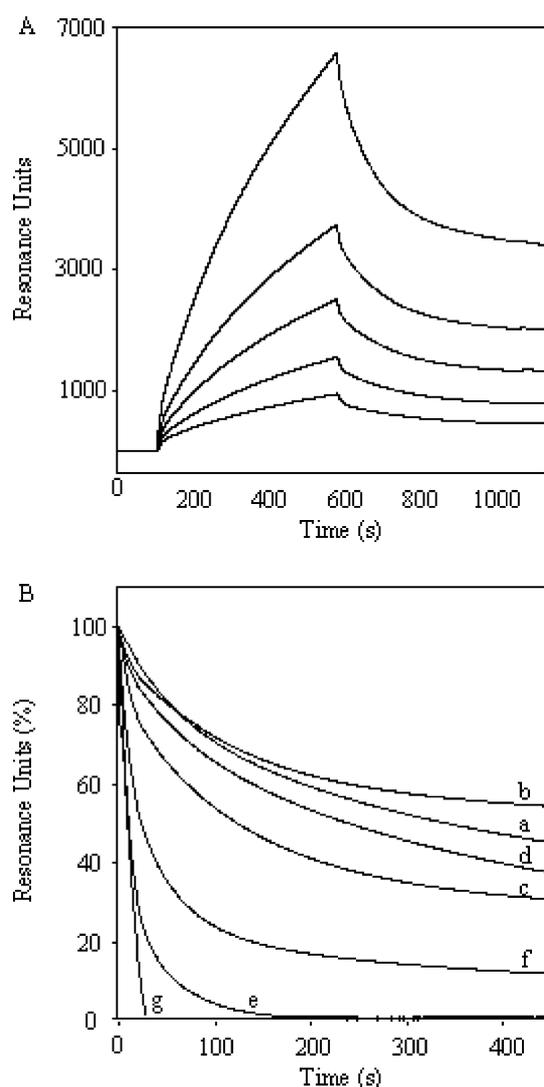


Figure 5. Analysis of binding kinetics by surface plasmon resonance on a BIAcore biosensor instrument. Overlay plots of sensorgrams obtained for scFv(1aa) at five different concentrations (4–28 µg/ml) against immobilised aGP (A) and of dissociation curves obtained for scFv constructs at 17 µg/ml against immobilised aGP (B). Curves a, scFv(0aa); b, scFv(1aa); c, scFv(2aa); d, scFv(3aa); e, scFv(4aa); f, scFv(9aa); g, scFv(18aa).

in Figure 5A. The kinetic data are summarised in Table 1. The scFv(18aa) exhibited very poor binding (maximal binding within the experimental settings was 125 resonance units), and illustrates the low affinity of this construct. All the dimeric constructs showed significantly improved binding kinetics in comparison to the scFv(18aa) but, surprisingly, to a different degree depending on the linker length. The scFv(3aa), which forms dimeric constructs just as scFv(2aa), scFv(4aa), and scFv(9aa), performed better in both ELISA and BIAcore assays, demonstrating that the mere formation of dimeric complexes does not automatically ensure the best binding kinetics. As expected, best binding was observed for the trimeric scFv(1aa) and the tetrameric scFv(0aa). It is intriguing that there were only small differences observed between scFv(0aa) and scFv(1aa). scFv(1aa) seemed to have a slightly slower dissociation rate than scFv(0aa), although the latter had higher valency, better overall binding (max RU), and appeared to be more stable when analysed by size-exclusion chromatography. As reported before, improved dissociation constants are often due to slower dissociation rates, which is clearly seen in an overlay plot of the sensorgrams (Figure 5B). The sensorgrams revealed a good correlation between the real-time binding kinetics and the direct ELISA analysis.

Affinity maturation

In an attempt to affinity mature the selected scFv, we generated five different libraries in which some of the CDRs were randomised by overlapping PCR. First, we targeted the HCDR3 by partial or complete randomisation. This was further extended to combinations with partial or complete randomisation of HCDR2 and/or LCDR3. Library size ranged from 2.5×10^6 to 2.5×10^8 and sequencing of randomly picked clones indicated good diversity ($N = 16$, respectively).

Selection strategies with increased selection pressure, such as diluted antigen concentration, prolonged washing steps or addition of competitor (scFv or TF), were investigated in the search for scFvs with higher affinities. However, in every case, all TF-specific binders identified encoded the same CDR-protein sequences as known from the founder

Table 1. Binding kinetics of scFv constructs obtained by BIAcore evaluation

	k_a (1/Ms)	k_d (1/s)	K_D (M)	RU (max) ^a
ScFv(0aa)	2.3×10^4	2.1×10^3	8.8×10^{-8}	9434
ScFv(1aa)	5.7×10^3	1.5×10^3	2.7×10^{-7}	6566
ScFv(2aa)	1.4×10^4	2.7×10^3	2.0×10^{-7}	3656
ScFv(3aa)	6.7×10^3	2.3×10^3	3.4×10^{-7}	5521
ScFv(4aa)	7.5×10^3	3.8×10^2	5.1×10^{-6}	860
ScFv(9aa)	1.1×10^4	4.7×10^3	4.3×10^{-7}	1918
ScFv(18aa) ^b	ND	ND	ND	125

Sensorgrams were obtained at five different concentrations (28 µg/ml, 17 µg/ml, 11 µg/ml, 7 µg/ml and 4 µg/ml) for all the constructs, and the binding kinetics evaluated with the BIAcore evaluation software.

^a RU (max) is the maximal bound antibody (in resonance units) after injecting a concentration of 28 µg/ml for 400 seconds.

^b The kinetics for the scFv(18aa) was not evaluated (ND) as the sensorgrams did not enable fitting with the software.

clone (data not shown). In addition, clones recognising peptide epitopes of aGP dominated the selections of library 5, in which all three CDRs were completely randomised.

Immunocytology

For a final proof of specificity and for detecting TF α in its natural tumor environment and density, the scFv(1aa) was tested in immunocytology with TF-positive and negative tumor cell lines. The cell line KG-1 (acute myelogenous leukemia), which consists of TF-positive (around 50%, varying from strong to weak) and negative cells¹⁴ showed the same characteristic TF-staining pattern of membranes when stained with scFv(1aa) as the tumor-specific murine IgM A78-G/A7 (Figure 6). Similar results were *mutatis mutandis* obtained with cell lines T-47D, HeLa SS6, and MDA-MB-231 (data not shown). Preliminary data demonstrate that the staining patterns of the murine and recombinant antibody are also fully comparable in immunohistochemistry of tumor sections (data not shown).

Discussion

Over the last decade we have tried a range of different strategies for generating TF-specific antibodies by the hybridoma technique. Several mice strains have been immunised with a panel of different TF-displaying immunogens such as asialofetuin, anti-freeze glycoprotein, aGP, TF-conjugated BSA, TF-conjugated peptides, LS 174T, T-47D, and KG-1 tumor cell lines, *Clostridium perfringens* cells, and finally neuramidase-treated T-47D cells and erythrocytes. In addition, several parameters in the immunisation protocol have been optimised such as dosage, number of boosts and various adjuvants. Out of these efforts only three hybridomas with the desired specificity were obtained. This illustrates the non-trivial task of

generating antibodies against this small non-charged disaccharidic structure. All three specific anti-TF antibodies originate from mice immunised with aGP and are of the IgM class. The latter is also true for the few other murine antibodies known from the literature that bind more or less specifically to TF.¹⁶⁻¹⁸

In the continuous search for TF-specific antibodies suitable for immunotherapeutic approaches we turned to the phage display technique, which has yielded large numbers of specific antibodies against many different targets.

Different proteins displaying TF such as aGP, asialofetuin, or TF coupled to BSA, and polyacrylamide conjugates³² with different densities of TF were initially used as target antigens in direct and/or competitive selection strategies on a panel of available naïve human scFv libraries (Griffin1, Tomlinson I and Tomlinson J). Direct selection strategies on immobilised antigens, biopanning on antigens in solution,³³ a combination of both, competitive selection strategies using the corresponding competitors (glycophorin, fetuine, BSA, or polyacrylamide conjugates), and specific elution, did not lead to the generation of TF-specific scFvs. The strategies failed although we used the proteolytically cleavable helper phage KM13, which is described to increase the efficiency of the selection²⁷ and allow the selection of larger varieties of scFv.²⁵ All scFvs obtained bound to the protein backbone, were cross-reactive to various other carbohydrates or recognised carbohydrate-dependent peptide epitopes (data not shown).

The only successful selection of antibodies from primary phage display repertoires against tumor-related oligosaccharides reported so far has been against the tetrasaccharide LeY, a larger carbohydrate structure against which also murine IgGs are available.^{34,35} We therefore reasoned that the lack of success in phage display selections against small non-charged tumor-specific carbohydrates

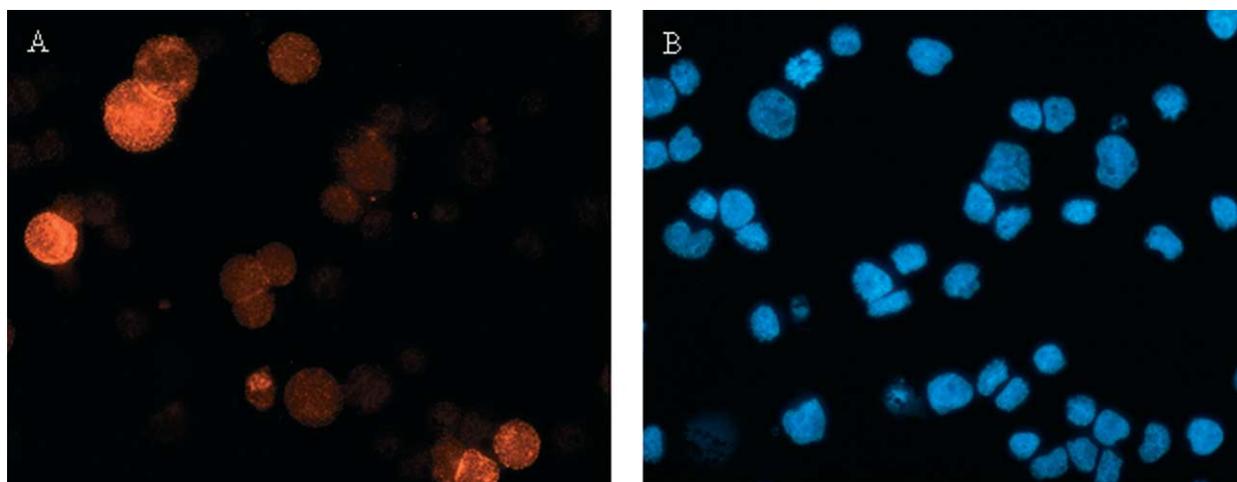


Figure 6. Immunofluorescence staining of KG-1 (acute myelogenous leukemia) cells with anti-TF multibodies. A, Staining with scFv(1aa), detected with mouse anti-His and anti-mouse-Cy3 antibodies; B, counterstaining of nuclei with DAPI. Magnification, 40 \times .

like TF could be due to a requirement for multivalent interactions. This is supported by the observation that multivalent display was essential in affinity maturation selections against a *Salmonella* carbohydrate.³⁶

Multivalency in phage display has been achieved using phage vector systems,^{37–40} but these systems have some drawbacks when the aim is to generate multivalent antibodies from immunised libraries. First, immunised libraries have a very narrow application range, therefore, their generation should not be time-consuming or labour-intensive. Second, the selected antibodies would be monovalent, and subsequent engineering would be necessary to obtain functional multivalent constructs. Phagemid vectors are more convenient for the generation of libraries from immunised donors. We focused on the construction of a new phagemid library format displaying multimers based on scFv with shorter linkers. In order to eliminate the background problem associated with phagemid systems and to allow fewer rounds of selection for maximum diversity we chose a combination with the novel KM13 helper phage.^{25–29} This combination allowed us to select functional multimers in solution directly suitable for *in vivo* applications which cannot be achieved through one of the recent pIII-deleted helper phage systems.^{41,42}

In comparative selections using scFv repertoires in the one and 18 amino acid residue linker format, respectively, only the scFv(1aa) libraries yielded TF-specific antibodies, confirming our hypothesis. The binding activities of monomeric, dimeric, or trimeric complexes of soluble scFv and the corresponding scFv constructs displayed on phage particles showed a clear correlation. Constructs with shorter linkers formed complexes of higher valency and binding activity in both systems.

Theoretically, multimeric constructs could form from several scFv-pIII-fusion proteins displayed on the same phage, or they could consist of a mixture of scFv-pIII-fusion proteins and non-fused scFvs. The presence of non-fused scFvs was demonstrated, but there seemed to be no significant difference in the amount of non-fused scFv among the different linker formats, while the overall display level was very similar for the different formats. We were therefore unable to demonstrate the exact molecular configuration causing the increased activity of shorter linker formats. However, the similarity of the binding activities of different linker formats seen in soluble form and on phage particles as well as the results of size-exclusion chromatography indicate that functional multimers are indeed formed on phages.

An essential finding was that all clones that specifically bound to TF encoded the same variable domain genes. The only differences found were in the primer-encoded regions, which were probably present in the library because several of the degenerate primers can amplify the same variable gene. Together with the results from affinity maturation experiments, which always led to the

selection of the founder clone, it suggests that the selected amino acid sequence is optimal for a TF-specific murine antibody. It also indicates that the sequence window for TF-specific antibodies is very narrow. The fact that no TF-specific IgGs have been identified from hybridoma fusions indicates that there is either no *in vivo* class switch to IgG, or that TF-specific IgGs were not identified due to their bivalent nature, which results in a lower functional affinity compared to IgM. The latter explanation is in agreement with the hypothesis that multivalency is needed for good binding of TF.

Mapping the fine-specificity of the scFv(1aa) in ELISA revealed almost exclusive binding to TF α independent of the carrier molecule. The binding is tumor-specific and relevant for the detection and efficient binding of TF in its natural tumor environment as shown by immunocytology and histology. At concentrations suitable for ELISA or immunohistochemistry, the scFv(1aa) bound only TF α with no cross-reactivity to other oligosaccharides tested. Only at very high concentrations was a limited cross-reactivity towards core-2-PAA, a trisaccharide composed of the TF structure and an additional GlcNAc, observed, which is shared by the tumor-specific antibody Nemo-TF2. This minor cross-reactivity to core-2 is not relevant for the tumor specificity, since core-2 is apparently always further elongated and therefore masked in tissues.

Molecular models of all possible Gal1-3GalNAc anomers and of core-2 (Figure 7) give a visual impression of the antigen and indirectly of the steric requirements for the binding pocket. They show the carbohydrate structure (space-filling model) connected to a serine residue (ball and stick model), and have been aligned so that the plane of the ring structure of GalNAc is in the same plane in the upper panel, and turned vertically by 120° in the lower panel. The antibody binding pocket seems to interact with the lower part of the carbohydrate molecules (Figure 7), where the non-bound anomers TF β (Gal β 1-3GalNAc β ; part of some gangliosides) and the non-natural structures Gal α 1-3GalNAc β and Gal α 1-3GalNAc α present steric hindrances in contrast to TF α and core-2. This can be clearly seen by comparing the right side of the models in the upper panel, where there is either a twist of the “overhang” for TF β or a change in the cavity below the overhang for the non-natural anomers (indicated by arrows) and when the models are turned 120° vertically as seen in the lower panel where a protruding structure is seen on the left side of the models of the non-natural anomers (arrows). Similar observations can be made with models of more complex structures which comprise the core-1 (non-exposed TF α) motif and are not bound by the scFv(1aa) (data not shown).

The binding kinetics of the scFv constructs was examined by surface plasmon resonance (Table 1). As expected, the tetrameric scFv(0aa) with its higher valency performed better in both ELISA and BIAcore analysis. Interestingly the off-rates of

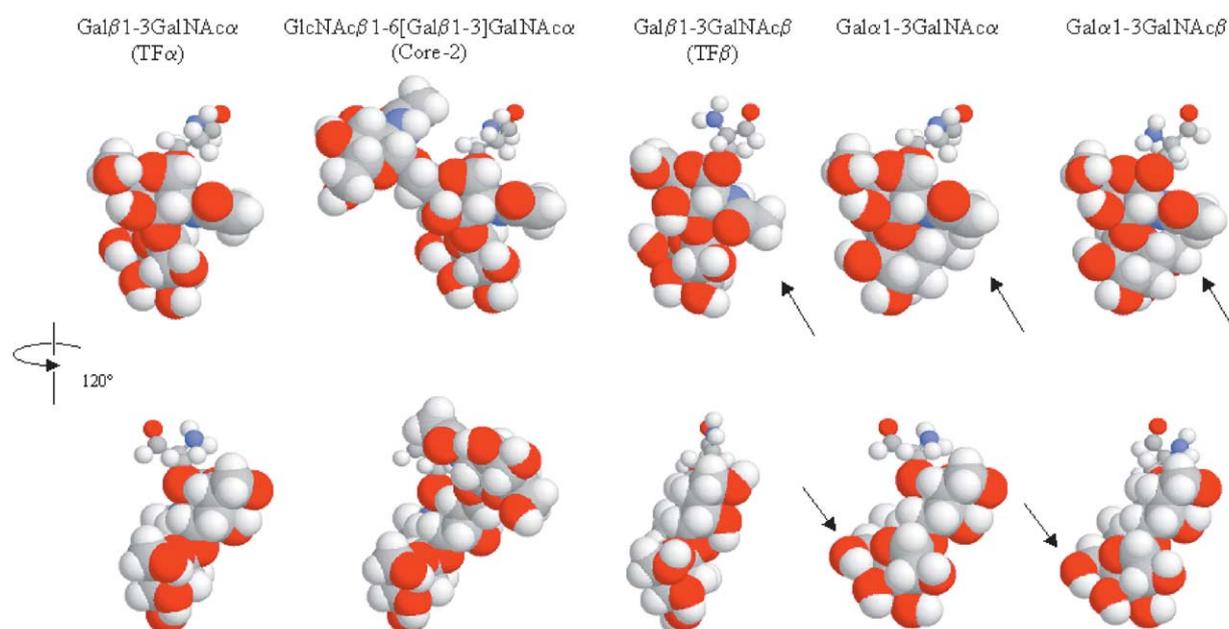


Figure 7. Models of carbohydrate structures. The carbohydrate structures are shown as (space-filling) models with a serine residue (as ball and stick) to indicate the location of the protein moiety. All models were aligned so that the ring structure of GalNAc is in the same plane in the upper panel, and have been turned 120° vertically in the lower panel. Arrows indicate steric differences in TF β , Gal α 1-3GalNAc α and Gal α 1-3GalNAc β compared to the tumor-relevant TF α and to core-2. Models were prepared with the SWEET2 software (www.dkfz-heidelberg.de/spec/sweet2/doc/).

the scFv(0aa) and the trimeric scFv(1aa) were very similar. The trimeric and tetrameric formats of the selected anti-TF scFvs are the choice for investigating the targeting potential in xenograft tumor models in the near future, which is not only due to the higher affinity but also to the increased size and the herewith expected favourable pharmacokinetics and biodistribution. The potential of trimeric constructs for clinical applications can also be reasoned by analogy from the pioneering studies on the anti-LewisY hu3S193 scFv, where the interest in multimeric complexes of scFvs also seems to increase.^{43–47}

In conclusion, we report here for the first time on the generation of recombinant antibodies toward a small, non-charged, and tumor-specific carbohydrate determinant (TF). The antibody was selected by phage display using a novel phagemid system displaying multimeric scFvs with increased functional activity. This system is expected to be of advantage for the generation of multivalent antibody fragments with clinical potential, antibodies toward antigens usually associated with low intrinsic affinity, and antibodies for internalisation where multimerisation of receptors plays a crucial role.

Materials and Methods

Bacteria

E. coli TG1 suppressor strain was used for the amplification of phages between rounds of panning.

E. coli BL21(DE3)pLysS and Rosetta™(DE3)pLysS

(Novagen, Germany) were used for the expression of the proteins.

Vectors

pET11a (Novagen) was modified to encode the PelB leader and the myc and His tags.

pHEN2 was kindly provided by Dr Greg Winter, Cambridge, UK.

Primers

All primers were synthesised according to the degenerate primer sequences reported by Zhou and co-workers;⁴⁸ however, the restriction site sequences in pHEN2 were adapted. Primer mixtures were always employed at concentrations of 10 μ M.

Primers introducing the BbsI site and used for the construction of scFv repertoires with single residue linkers are listed in Supplementary Material, Table 2.

Immunised library construction

Three Balb/c and two C3H/J mice were immunised intraperitoneally (i.p.) with 100 μ g of aGP (Sigma, Taufkirchen, Germany) per mouse together with complete Freund's adjuvant (CFA), followed 24 hours later by 100 mg/kg of cyclophosphamide (Sigma) as described by Matthew *et al.*,⁴⁹ boosted two weeks later with 100 μ g of aGP without CFA, and sacrificed four days after boost. The spleen cells from one Balb/c mouse and one C3H/J mouse were used in a separate attempt to generate hybridomas. Spleen cells from one Balb/c mouse and one C3H/J mouse were used for RNA extraction. Simultaneously, a fraction of the splenocytes was cultivated in RPMI 1640 medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% (v/v) FCS (Gibco)

and 5% HCF (Origen hybridoma cloning factor, TEBU, Frankfurt/M, Germany) in order to test the culture supernatants for antibody response in ELISA.²⁹ The culture supernatant from the C3H/J splenocytes used for RNA extraction indicated the presence of TF-binding antibodies, whereas the supernatants from the other mice were negative (data not shown). The spleen cells from the remaining Balb/c mouse were cultivated for two days before RNA extraction. Half of these cells were cultivated in the presence of 2 µg/ml of aGP for a potential specific stimulation.

Total RNA was extracted from ~10⁷ cells using the RNAeasy Midi kit (Qiagen, Germany), and eluted in 300 µl of diethyl pyrocarbonate (DEPC)-treated water. Secondary structures of the RNA were destroyed by heating to 65 °C for two minutes, and the mRNA captured on oligo(dT)25 Dynabeads (DynaL Biotech, Hamburg, Germany) in a final volume of 600 µl of mRNA binding buffer (10⁸ beads in 20 mM Tris-HCl (pH 7.5), 1.0 M LiCl, 2 mM EDTA). After rotating ten minutes, the beads were collected and washed twice with 1 ml of mRNA washing buffer (10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA) at room temperature and twice with 1 ml of ice-cold DEPC-treated water. The captured mRNA was reverse-transcribed to ss-cDNA using the oligo(dT)25 beads as primer and 16 units of Omniscript reverse transcriptase (Qiagen), 0.5 mM dNTPs and 40 units RNase inhibitor in a total volume of 80 µl by incubation at 37 °C for two hours.

Variable heavy chain genes were amplified by PCR using V_H-For(1aa) primer mix and V_H-Back primer mix and all the ss-cDNA coupled beads as template in a total volume of 400 µl. Subsequently, the cDNA coupled beads were retrieved, washed in TE, and applied as template in the PCR amplification of the variable light chain genes using the V_L-For primer mix and V_L-Back(1aa) primer mix.

The amplified genes were purified using the Qiagen PCR purification kit, and digested overnight with BbsI. The digests were separated on a 2% (w/v) agarose gel, and the desired DNA fragments excised and extracted. V_H and V_L genes were joined by ligation (T4 DNA ligase; NEB, Germany) and amplified by PCR using the V_H Back and V_L For primers. Finally, the PCR product was purified and cloned into pHEN2 using NcoI and NotI, resulting in repertoires of average size 5 × 10⁶ Da.

Libraries in the scFv(18aa) format were cloned into pHEN2 using standard procedures.

Library amplification and selection

Phage libraries or single phage clones were always prepared fresh from -80 °C master stocks as described.²⁵⁻²⁷ Selections were carried out using approximately 5 × 10¹² cfu, which were pre-incubated in 1% BSA-PBS for 30 minutes at room temperature. Selections in immunotubes (Nunc, Roskilde, Denmark) were performed on aGP and biopannings were performed in solution with biotinylated antigen and capture with streptavidin-conjugated magnetic beads (DynaL) as described.²⁵⁻²⁷ (See Supplementary Data for more detail.)

Cloning of secondary libraries for affinity maturation

The primers for randomisation of the HCDR2, HCDR3 and LCDR3 were designed to encode sequences flanking the CDRs on both sides, whereas the CDR sequences were randomised partially (70% bias for the original sequence) or completely (including length for HCDR3 and LCDR3).

ScFv gene fragments encoding the randomisations were PCR amplified, and the scFv genes reassembled by overlapping PCR extension. Finally, the scFv libraries were ligated into pHEN2 with restriction enzymes NcoI and NotI and transformed into *E. coli*.

Construction of multimers

The scFv(0aa), scFv(2aa) and scFv(3aa) constructs were generated by overlapping PCR extension or normal "cut and paste" cloning as described in the Supplementary Data.

Sequencing

Sequences were obtained as Extended Hot Shot from Sequence Laboratories (Göttingen, Germany).

Phage ELISA screening

Monoclonal phage particles were screened for binding in ELISA as described by Marks⁵⁰ (see the Supplementary Data for more detail).

ELISA

Coating, washing, development and quenching were performed as described for the phage ELISA screening (standard coating concentrations were 10 µg/ml). Antibodies and antibody fragments were applied as primary reagents in 100 µl 1% BSA-PBS and incubated for one hour. Tetra-His HRP conjugate (Qiagen) 1:1000 in 1% BSA-PBS was used as secondary reagent for detection.

Protein expression and purification

Proteins were expressed in *E. coli* Rosetta™(DE3)pLysS (Novagen) using a modified pET11a expression vector (Novagen), and purified by standard IMAC (see the Supplementary Data for further information).

Western blots

Western blots were performed with anti-myc antibodies 9E10 (Sigma), Tetra-His (Qiagen) or anti pIII (MoBiTec, Göttingen, Germany) as recommended by the manufacturers. As secondary reagent, we applied an anti-mouse/HRP conjugate (Dako), and developed the blots using a chemiluminescence kit (SuperSignal, Pierce, USA).

Surface plasmon resonance studies

Measurements were performed on a BIA sensor machine (BIAcore2000, Pharmacia) in PBS (pH 7.0) with a flow rate of 5 µl/minute at 25 °C. aGP was immobilised covalently on the dextran matrix of a CM5 sensor chip using the standard amine immobilisation procedure according to BIAcore protocols. A high surface density of aGP (16,800 RU) was immobilised. For analysis of the scFv constructs, sensorgrams at five different concentrations (28 µg/ml, 17 µg/ml, 11 µg/ml, 7 µg/ml and 4 µg/ml) were obtained. Dissociation was analysed by injection of PBS. After each measurement, the chip surface was regenerated with two volumes of 10 µl of PBS (pH 11) followed by PBS (pH 7.0).

Immunocytology

Cells were cultured on multitest slides (ten wells, Roth, Karlsruhe, Germany) for 24 hours. After careful removal of the medium, slides were air-dried, wrapped, and stored at -80°C . After thawing, cells were briefly fixed with formalin (acid-free for histology, Merck, Darmstadt, Germany; 5% in PBS, five minutes), and incubated with scFv(1aa) (10 $\mu\text{g}/\text{ml}$ in PBS, 90 minutes at 4°C). Second antibodies were either mouse tetra-His/HRP or anti-c-myc 9B11 antibodies (1:1000), followed by goat anti-mouse immunoglobulin (H+L), Cy3-labeled (Dianova, Hamburg, Germany; 1:100), and finally DAPI (0.5 $\mu\text{g}/\text{ml}$, one minute). Slides were mounted with glycerol-buffer and a trace of *p*-phenylenediamine, and examined with an Axioplan 2 microscope (Zeiss, Jena, Germany).

Acknowledgements

The work was supported financially by the Danish Research Training Council (Forskerudannelsesrådet), NEMOD Biotherapeutics GmbH & Co.KG and GLYCOTOPE GmbH. The authors thank Dr Greg Winter (MRC, Cambridge, UK) for kindly providing phage antibody libraries and vector, Dr Ian Tomlinson (MRC, Cambridge) for providing the Tomlinson I and J repertoires, and Dr Olav Andersen for supervision on the BIAcore experiments.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2004.08.052](https://doi.org/10.1016/j.jmb.2004.08.052)

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Edited by J. Karn

(Received 1 June 2004; received in revised form 9 August 2004; accepted 13 August 2004)