Cancer Immunol Immunother (2008) 57:367–378
DOI 10.1007/s00262-007-0376-2

ORIGINAL ARTICLE

Isolation of anti-MISIIR scFv molecules from a phage display library by cell sorter biopanning

Qing-An Yuan · Matthew K. Robinson · Heidi H. Simmons · Maria Russeva · Gregory P. Adams

Received: 9 May 2007 / Accepted: 13 July 2007 / Published online: 4 August 2007

Abstract While cell surface antigens represent the most common targets for antibody-based cancer therapy, isolation of new antibodies specific for these targets from single-chain Fv phage display libraries has been hindered by limitations associated with traditional selection techniques. Solid phase panning is often associated with conformational changes to the target protein due to its immobilization on plastic tubes that can limit the ability of the isolated scFv to bind to conformational epitopes and solution panning methods require the use of secondary tags that often mask desired sequences and create unintended epitopes. Commonly utilized cell-based panning methods typically yield a panel of single-chain Fv (scFv) molecules that are specific for numerous cell surface antigens, often obscuring the desired clones. Here, we describe a novel cell sorter-based system to isolate single-chain Fv molecules specific for defined antigen targets expressed on stably-transformed mammalian cells. We employed these methods to isolate promising scFv clones that bind specifically to the Müllerian inhibiting substance type II receptor, a cell surface ovarian cancer antigen that has proven to be a difficult target for selection strategies.

Keywords Antibody · Cell sorting · Phage display · Müllerian inhibiting substance type II receptor · Single-chain Fv

Q.-A. Yuan · M. K. Robinson · H. H. Simmons · M. Russeva · G. P. Adams (✉)
Department of Medical Oncology,
Fox Chase Cancer Center, 333 Cottman Ave,
Philadelphia, PA 19111, USA
e-mail: gp_adams@fccc.edu; gregory.adams@fccc.edu

Introduction

Antibodies targeting relevant cell surface antigens are playing an increasingly important role in cancer diagnosis and therapy [2, 8, 43]. Due to tolerance of self antigens and limited effectiveness of human hybridoma fusion partners, most monoclonal antibodies (MAb) currently in the clinic were initially developed by immunizing mice, generating murine hybridomas [28] and humanizing promising MAbs by CDR grafting [26]. More recent generations of MAbs entering clinical trials are derived from transgenic mice with human immunoglobulin genes [12] or phage display of human immunoglobulin variable domains in single-chain Fv (scFv) or Fab formats [53]. As each phage contains the gene encoding the scFv or Fab on its surface, straightforward panning techniques can be used to isolate clones specific for a desired antigen assuming it is available in a highly pure form. These techniques are fast and efficient and typically use target antigens in a "solid phase" that are coated onto a plastic support (e.g., immunotubes or microtiter plates) or in solution employing tagged antigens that can be isolated using magnetic beads coated with a ligand specific for the tag [10]. Phage display has also been employed to a lesser extent to isolate antibody clones specific for unknown antigens present on the surface of the desired target cell population [30]. While this strategy often yields antibodies specific for a wide range of undesired common and uncommon cell surface proteins, strategies can be employed to focus the specificity of the resulting clones. These include performing an initial panning step against a normal human cell population to deplete undesired clones prior to panning against the tumor cell line of interest [41] and stripping unwanted phage from the cell surface prior to recovering the selected phage to isolate those that target internalized antigens [23, 40].

Most selection strategies use the solid phase methods described above. While these are often effective, the process of coating the protein target onto plastic frequently causes significant changes in its structure [5, 6], often resulting in the isolation of antibody clones that are not capable of recognizing the antigen in its native conformation. Additionally, selections performed against recombinant forms of a cell surface protein (e.g. a growth factor receptor) often yield clones to epitopes that are either not exposed due to protein conformation or not accessible due to steric hinderance by other components in the cell membrane [37]. Cell-based panning strategies that present the target antigen in a more natural conformation could potentially yield antibody clones with a higher specificity and accessibility for the desired target if the binding to background antigens could be curtailed. However, the current methodologies involve complicated optimization of choosing appropriate cell lines, the number of cell number used, incubation duration, washing condition etc. to achieve acceptable signal/noise ratio and decent positive yield [51].

In this report we describe a novel method to overcome the obstacles to cell based panning that are mentioned above. For these studies we have utilized the human Müllerian inhibiting substance [36] type II receptor (MISIIR), which is also known as the anti-Müllerian hormone (AMH) receptor type II (AMHRII), as a target antigen. MISIIR is involved in the regression of the female Müllerian duct during the normal development of the male reproductive system (reviewed in [38]) and is reported to be expressed in more than 50% of human ovarian carcinomas ascites cells [33] with a very limited distribution pattern in normal female tissues [4, 22, 49]. Significantly, primary cellular isolates from the ascities of patients with ovarian cancer and numerous ovarian cancer cell lines underwent apoptosis when treated with the receptor's natural ligand, MIS [9, 15, 16, 18, 33, 48]. In our opinion, this makes MISIIR an attractive target for antibody-based intervention of ovarian cancer. The ideal anti-MISIIR antibody would be one that mimics the specificity and cytotoxic effects of MIS. Our initial attempts to isolate anti-MISIIR scFv clones from three different non-immune libraries utilized a recombinant human MISIIR ECD:Fc domain fusion protein in both solid phase and solution panning strategies. These procedures yielded a number of unique clones that bound specifically to the MISIIR ECD:Fc fusion protein in ELISAs but not to other fusion proteins containing human Fc domains [54]. However, many of the clones that exhibited positive binding in ELISAs failed to bind to native MISIIR on the surface of tumors cells in flow cytometry assays. To overcome this obstacle, we developed the cell sorter-based methodology described here.

Materials and methods

Human scFv phage library construction

A large naïve human single chain variable fragment (scFv) phage display library was constructed essentially as previously reported [46]. Briefly, whole blood (50 ml/donor) was collected from 47 healthy donors and lymphocytes were isolated by Ficoll-Paque gradient [14]. Total RNA was extracted from individual samples (Cat#74104, RNeasy®, QIAGEN, Valencia, CA, USA) and subjected to reverse transcription (5 μg RNA/rxn) with random hexamer primers. Variable heavy (VH) and light (Vκ and Vλ) genes were amplified from each donor by nested PCR using HotStarTaq (Qiagen, Cat# 203443) and primers based upon human antibody sequences (http://www.bioc.unizh.ch/antibody/Sequences/index.html). Outside primers (Table 1) were designed to match the leader regions of the VH, Vκ or Vλ chains and constant regions of both heavy and light chains. Inside primers were designed to match the 5′- and 3′-terminal consensus sequences, respectively, and to insert restriction sites. To facilitate directional cloning of the VH and VL domains, inside VH primers incorporated unique *Sfi*I sites at both ends following the technique reported by Krebber et al. [31]. Inside VL primers incorporated *Spe*I and *Xho*I restriction sites at the 5′ and 3′ ends of the amplified fragments. Amplified fragments were gel-purified (Qiaquick Gel Extraction Kit, Cat# 28706, QIAGEN), VH, Vκ and Vλ mixes representing the entire diversity of the donors were created by combining the purified fragments, and subjected to restriction digestion with above mentioned enzymes, as appropriate.

To construct the scFv library, pAK100 [31] kindly provided by Andreas Pluckthun, University of Zurich, was modified to create pAK100-lnk by the addition of a $(Gly_4Ser)_3$ linker sequence and *Xho*I and *Spe*I restriction sites. A two-step cloning method was then employed to assemble a library of intact scFv. First a VL sublibrary was constructed by subcloning Vκ and Vλ mixes (1:1 molar ratio) into pAK100-lnk. Ligated DNA was electroporated into TG1 *Escherchia coli* (Cat# 200123, Stratagene. La Jolla, CA, USA) and cells were cultured in NE (Non-Expression medium, 2× YT with 1% glucose and 25 μg/ml chloramphenicol) medium to 1.0 OD_{600}. Plasmids corresponding to the VL sublibrary were extracted *en masse*, digested with *Sfi*I (New England Biolabs, Ipswich, MA, USA), gel purified, ligated with purified VH fragments, and ligated DNAs were electroporated into TG1 *E. coli*. Fifty electroporations (approximately 1 μg per reaction) were performed, the cultures combined, and a serial dilutions plated to determine the approximate size of the final scFv library. The combined culture was expanded in NE media, grown to 1.0 OD_{600}, and aliquots of culture were frozen as

Table 1 "Outside" primers used for primary amplification of human naïve antibody variable domains and flanking regions

Backward outside primers for VH leader regions	
HLd1	5′-TGG AGG/C,A A/GTC CTC/T TTC/T TTG GTG G-3′
HLd2	5′-C ACG/A CTC CTG CTG CTG ACC A-3′
HLd3A	5′-CT ATT/A T/ATA/T G/A,CAA GGT GTC CAG TGT-3′
HLd3B	5′-GCT CTT TTA AGA GGT GTC CAG TGT-3′
HLd4	5′-GTG GCA/G GCT CCC AGA TGG GTC CTG-3′
HLd5	5′-CTG GCT GTT CTC CAA GGA GTC TGT-3′
HLd6	5′-CTG GGC CTC CCA TGG GGT GTC CTG-3′
Backward primers for Vκ leader regions	
kLd1	5′-GCT CAG CT/GC CTG GGG/A CTC/T CTG C-3′
kLd2	5′-TC/T CTG GGG CTG CTA ATG CTC TGG-3′
kLd3	5′-TC TTC CTC CTG CTA CTC TGG CTC-3′
kLd4	5′-T CTG TTG CTC TGG ATC TCT GGT G-3′
kLd5	5′-GC TTC CTC CTC CTT TGG ATC TCT G-3′
kLd6	5′-CTC TGG GTT CCA GCC TCC AGG GGT-3′
Backward primers for Vλ leader regions	
λLd1	5′-T CAC TGT/C A/GCA GGG TCC TGG GCC-3′
λLd2	5′-ATG GCC TGG GCT CTG CTC/G CTC CTC-3′
λLd3	5′-ATG GCA/C TGG AT/CC CCT CTC TTC-3′
λLd4A	5′-CCC TTC ATT TTC TCC ACA GGT CTC-3′
λLd4B	5′-CTC CTC CTC CAC TGC/G ACA GGT TCT C-3′
λLd5	5′-TC CTC TCT CAC TGC ACA GGT TCC C-3′
λLd6	5′-CTC GCT CAC TGC ACA G/GT TCT TGG-3′
λLd7	5′-G TTC CTC CTC ACT TGC TGC CCA GG-3′
λLd8	5′-C GGA CTC CTT GCT TAT GGA TCA GG-3′
λLd9/10	5′-CC/TG/T CTG/C CTC CTC/G ACC CTC CTC A-3′
Forward out-side primers for constant region	
CHFOR	5′-TGG AAG AGG CAC GTT CTT TTC TTT-3′
CκFOR	5′-ACA CTC TCC CCT GTT GAA GCT CTT-3′
Cλa-FOR	5′-TGA ACA TTC TGT AGG GGC CAC TG-3′
Cλb-FOR	5′-AGA GCA TTC TGC AGG GGC CAC TG-3′

seeds for future production of phage libraries. Propagation of combinatorial phage libraries rescued by M13K07 helper phage (New England Biolabs) was performed as published procedures [31]. Phage antibody titer was measured and library aliquots were stored at −70°C in a solution of 50% glycerol.

Construction of MISIIR ECD expressing cell line

A cDNA encoding the MISIIR ECD was isolated previously [54] and served as the source of DNA to create a cell line that stably expressed MISIIR ECD on its surface. Briefly, *Bgl*II and *Pst*I restriction sites were incorporated at the 5′ and 3′ ends of the MISIIR ECD cDNA by PCR using the pDISfor (5′<GGG AGA TCT CCC CCA AAC AGG CGA ACC TGT G>3′) and pDISrev (5′<GGC TGC AGG GAC TCA CCT GGG GCA GCC TGG>3′) primers. The resulting fragment was subcloned into pDisplay (Invitrogen, Cat # V660-20). The resulting pDisplay-MISIIR expression vector encodes a translational fusion between the MISIIR ECD and the platelet derived growth factor receptor (PDGFR) transmembrane domain with a Hemagluttin (HA) epitope tag at the N-terminus of the fusion protein to facilitate monitoring of expression. The construct was linearized with *Pvu*I (New England Biolabs), transfected into HEK293 cells (Cat# CRL-1573, ATCC) using Fugene 6 (Roche Diagnostics, Basel, Switzerland), and stable transfectants were selected with 1000 μg/ml G418 antibiotic (Cat# MT-30-234-CR, Cellgro, Herndon, VA, USA). A clone that expresses moderate to high levels of MISIIR ECD (hereafter referred to as HEK::MISIIR"+") was isolated by cell sorting on a FACS-VantageSE/DiVa instrument (Becton Dickinson, Franklin Lakes, NJ, USA) using fluorescin-conjugated anti-HA antibody 3F10 (Roche Diagnostics GmBH, Mannheim, Germany). A corresponding MISIIR-negative control cell line (hereafter referred to as HEK::MISIIR"−") was established by transfecting the linearized parental pDisplay vector as detailed above. Both

HEK::MISIIR"+" and HEK::MISIIR"−" cell lines were grown in DMEM supplemented with 10% female FBS (Biotechnics Research Inc., Lake Forest, CA, USA), G418 (1,000 μg/ml) and penicillin-streptomycin and L-glutamine (Invitrogen) in 5% CO_2 at 37°C. Selective expression of MISIIR ECD on the surface of HEK::MISIIR"+" cells as compared to parental HEK293 cells by flow cytometry using the 12G4 murine anti-MISIIR monoclonal antibody (kind gift of Dr. Navarro-Teulon, [44]) and FITC-conjugated goat anti-mouse IgG (Cat # 349031, BD Biosciences, San Jose, CA, USA) as a secondary antibody. The secondary alone failed to bind either HEK293 or HEK::MISIIR "+" cells (data not shown).

Cell/phage incubation and sorting

HEK::MISIIR"+" cells were uniformly labeled with Cell Tracker Green (Cat # C2925, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with minor modifications. Briefly, HEK::MISIIR"+" cells were grown to 70% confluence, washed once with Phosphate Buffered Saline (PBS) to remove non-adherent cells, and harvested by incubation in Hanks's Balanced Salt Solution (HBSS, Invitrogen) containing EDTA (1 mM). A single cell suspension was obtained by passage through a cell strainer (Cat# 352340, BD Falcon™, BD Bioscience, San Diego, CA, USA) and cells were stained at 1.2×10^6 cells/ml in pre-warmed staining solution (serum-free DMEM containing 10 nM Cell Tracker green BODIFY) for 20 min at 37°C. Staining solution was replaced by 1 ml prewarmed (37°C) serum-free medium (Cat# 22600-050, OPTI-MEM®I, Invitrogen) and cells were incubated for an additional 30 min at 37°C. Cells were then washed once in 1 ml PBS, and blocked in 1 ml of 4% MPBS (Milk PBS; 4% w/v fat-skimmed milk powder in PBS) at room temperature for 30 min.

HEK::MISIIR"−" cells ($1–2 \times 10^7$) were incubated at room temperature with the scFv-phage library (0.2 ml, 2×10^{13} cfu/ml) for 1 h in 2 ml 4% MPBS. HEK::MISIIR"+" cells (2×10^4) stained with Cell Tracker Green, as detailed above, were then added and the cell/phage mixture was incubated on a rotator at room temperature for another 2 h. Cells were pelleted (500×*g*, 5 min), resuspended at 2×10^7 cells/ml in PBS and HEK::MISIIR"+" cells were collected by cell sorting on a FACSvantage SE cell sorter (Becton-Dickson) based on fluorescence at 488 nM. Cells were collected in a tube containing 100 μl PBS to prevent desiccation and then bound phage were eluted by incubation with 900 μl fresh-made 100 mM TEA (triethylamine, Cat# 90337, Sigma St. Louis, MO, USA) at RT for 10 min. The solution was neutralized with 500 μl Tris–HCl (1 M, pH 7.4) and half of phage solution was used to infect log-phase TG1 which was then plated on NE dish. Bacteria lawn was scraped from the plates, either frozen with 10% glycerol or used as inoculum for further rounds of phage preparation.

Phage-antibody preparation

Concentrated phage antibodies were prepared as described [31]. Briefly, bacteria were inoculated into a 250 ml baffled flask containing 50 ml NE medium and grown at 37°C, 250 rpm to an OD600 = 0.5. Phage were rescued from 10 ml of culture by the addition of with 1×10^{11} pfu (Plaque-forming unit) M13K07 helper phage and 5 μl IPTG (isopropyl-beta-D-thiogalactopyranoside, 1 M stock; Cat# 16758, Sigma). Cells were incubated at 37°C without agitation for 30 min, the culture was expanded by the addition of 40 ml of LE (2XYT containing 1% glucose, 25 μg/ml chloramphenicol, 0.5 mM IPTG) medium, and grown overnight (>8 h) at 26°C, 250 rpm to induce production of phage antibodies. Bacterial cells were pelleted from 40 ml of the culture and phage were precipitated with 1/5 volume 20%PEG/2.5 M NaCl (PEG-6000, Cat# P-2139, Sigma) by centrifugation at 3,300×*g* for 30 min. The phage pellet was dissolved in 1 ml PBS and centrifuged at 11,500×*g* for 10 min to remove bacterial debris. The clarified phage antibodies were used immediately or frozen at −80°C in 10% glycerol for future use.

Monophage ELISA, PCR fingerprint and scFv expression

Monophage ELISA was carried out essentially as described [27, 54]. Positive clones were amplified by PCR using the primers GA213 (5′>TCATGAAATA CCTATTGCC TACG<3′) and GA215 (5′>CCAGAGCCACCACCCTA CA GGT<3′) and categorized by PCR fingerprinting [32]. Unique colonies were then identified by sequence analysis. Expression of soluble scFv was accomplished by subcloning the genes for the scFv from the pAK100-lnk phagemid to pCYN2 [45] as *Nco*I and *Xho*I fragments. Soluble scFv were expressed in *E.coli* TG1, isolated from the periplasmic space and purified by Ni-NTA agarose affinity chromatography and high-performance liquid chromatography (HPLC) as previously described [1]. The size and integrity of the resulting scFv were assayed by 12% SDS-PAGE [34].

scFv:Fc preparation and affinity determination by SPR

Construction and preparation of scFv:Fc was performed as described [54] with minor modifications. Briefly, the 3′-*Sfi*I sites of selected scFv genes were mutagenized (Quik Change, Cat # 200514, Stratagene, La Jolla, CA, USA) and the genes were subcloned into a modified pHingestuffer [19] for expression and purification. Binding of scFv and scFv:Fc to purified MISIIR ECD:Fc was carried out by

surface plasmon resonance on a BIAcore1000 (BIAcore, Pharmacia, Piscataway, NJ, USA) as previously described [54].

Flow cytometry

AN3Ca (Cat# HTB-111, ATCC, Manassas, VA, USA) human endometrial adenocarcinoma cells (2×10^5) were harvested from logarithmically growing cultures and washed with FACS buffer (1% BSA in PBS, 0.02% NaN3). Cells were then incubated with 1 μg of scFv:Fc and for 30 min on ice, washed twice with FACS buffer and then incubated with a FITC-labeled anti-human IgG MAb (Cat# 05-4211, Biosource, Camarillo, CA, USA). Binding to cells was analyzed by flow cytometry on FACscan flow cytometer (Becton & Dickson) and data were analyzed using CellQuest Pro software program (Becton & Dickson).

Evaluation of in vitro GY4 scFv/Fc directed immunotoxin activity

The assay was performed similarly as described [29]. Logarithmically growing AN3Ca (MISIIR"+", EGFR"+", HER2"−") and COS7 (human MISIIR"−", human EGFR"−", human HER2"−") cells were harvested and resuspended to 2.5×10^4 cells/ml in MEM supplemented with 2 mM L-Glutamine, 10% female FBS and penicillin (100 μg/ml)- streptomycin (100 units/ml). Cells (2,250 cells/well, 90 μl) were dispensed into 96-well plates (Costar®, Cat# 3593, Corning Incorporated, Corning, NY, USA) and allowed to adhere for 24 h at 37°C in the presence of 5% CO_2. Hum-ZAP (Cat# IT-22-25, Advanced Targeting Systems, San Diego, CA, USA) was resuspended in media to a concentration of 3.3×10^{-8} M and 10 μl (50 ng) was added to each well followed by 100 μl of an appropriate 2× stock of GY4 scFv:Fc, ABX-EGF (panitumumab, Amgen, Thousand Oaks, CA, USA), trastuzumab (Herceptin®, Genentech, San Francisco, CA, USA), or media controls. ABX-EGF (anti-EGFR) and trastuzumab (anti-HER2) are FDA-approved for the treatment of metastatic colorectal and breast cancer, respectively. The final concentration of Hum-ZAP in all assays was 0.25 nM with targeting antibodies at 100, 10 or 1 nM. Excess targeting antibody was used to promote incorporation of the Hum-ZAP into complexes with the targeting antibodies. The plates were incubated for 96 h at 37°C in the presence of 5% CO_2 and then cell growth was measured with CellTiter 96 Non-Radioative Cell Proliferation Assay, (Cat# 4000, Promega Corp. Madison, WI, USA) according to the manufacturer's protocol. Plates were read at 570 nm in a Multiskan Ascent plate reader (Labsystems Ltd, Kennett Square, PA, USA). Each assay was performed at least three times with similar results. Statistical analysis: unpaired *t* tests were performed using the online calculator available at http://www.graphpad.com/quickcalcs/ContMenu.cfm. P values ≤ 0.05 were considered to be statistically significant.

Results

Phage display library

A large, naïve, human scFv phage display library was created from the immunoglobulin variable domains of 47 healthy donors using a two-step cloning strategy. Donors from a variety of ethnic groups and nationalities, including African American, Caucasian, Chinese, Hindu, native American, Japanese, and Jewish, were specifically incorporated into the library in an attempt to enhance the range of variable domain polymorphisms present within the scFv library. The cDNAs amplified from each individual were pooled and the mixture was used in nested PCR reactions with primers specific for the nine VH, seven Vκ, and eight Vλ gene subfamilies. Equal molar ratios of each PCR product from the Vκ and each Vλ subfamilies were sublconed into pAK100-lnk to generate a 6×10^7 cfu VL library. Subcloning the VH families (into the VL sub-library) resulted in a final scFv library that contained an estimated 2.3×10^{10} independent transformants. Sequencing of 34 randomly selected scFv clones predicted that the library contained 82% full-length scFv inserts with approximately 73% of the total clones encoding for functional open reading frames. Despite the limited number of sequences evaluated, gene family analysis of showed that six out of the nine VH gene families were represented. VH1 comprised 34% of the clones with VH6 representing an additional 22% of the clones. The remainders of the clones were comprised of VH2, VH3, VH4, and VH5.

Development of a cell-based selection protocol

Panning scFv-phage libraries by conventional cell-based methodologies is generally associated with high background and a low yield of positive clones to the desired antigen. We hypothesized that antigen-negative cells could be used in a cell-based panning strategy to deplete background binding of phage while selection of antigen-positive cells via a cell sorting strategy, that is independent of phage binding, could be used to increase the yield of scFv-phage specific for the desired antigen. As a proof-of-concept for this strategy we established two HEK293-based cell lines that differ only by the expression of our target antigen, the MISIIR ECD. This was accomplished by selecting HEK293 cells that stably express high levels of the MISIIR ECD as a fusion protein with the transmembrane domain of the platelet derived growth factor receptor (PDGFR). The cell line, HEK::MISIIR"+", was isolated by FACS with a

MAb specific for the HA-tag found on the N-terminus of the fusion protein. Specific expression of MISIIR ECD on the surface of HEK::MISIIR"+" cells, as compared to parental HEK293 cells, was confirmed by flow cytometry with a monoclonal antibody specific for the HA-tag (Fig. 1a) and a Monoclonal antibody specific for human MISIIR (Fig. 1b, [44]). Separation of HEK::MISIIR"+" cells from an excess of HEK::MISIIR"−" cells was accomplished by cell sorting. Figure 2 demonstrates that HEK::MISIIR"+" cells stained with the vital dye CellTracker Green can be readily separated from unstained HEK::MISIIR"−" cells despite representing only 0.02% of the total cell number in the experiment.

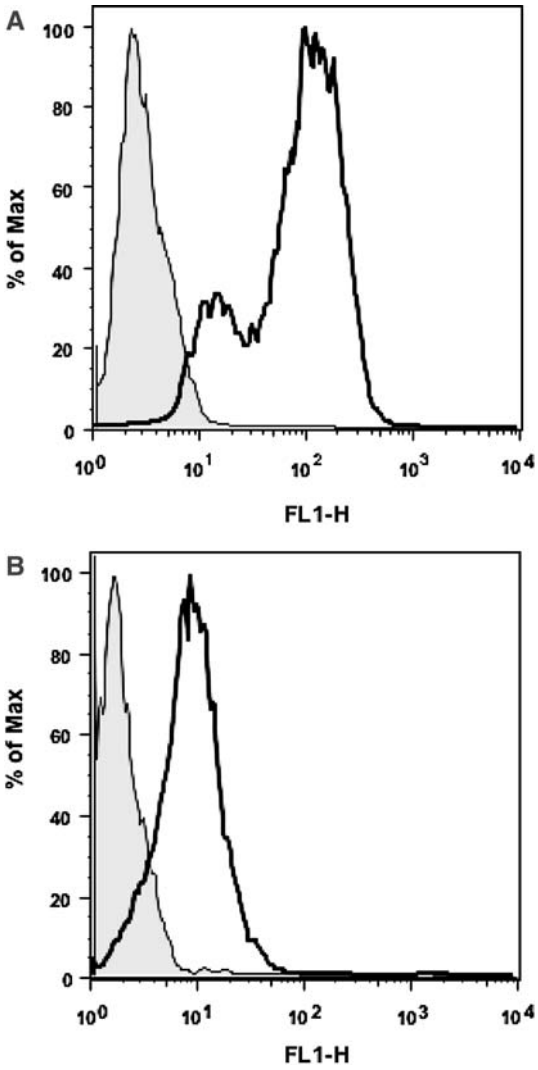

Fig. 1 Flow cytometry analysis of MISIIR ECD expressing HEK:MISIIR"+". Following the stable transfection of HEK cells with the pDisplay vector encoding the MISIIR ECD gene and a N-terminal HA tag, specific expression of the HA tag and the MISIIR ECD on the cell membrane was determined by flow cytometry. **a** Expression of HA-tagged MISIIR ECD on the surface of HEK::MISIIR"+" cell line (*unfilled line*) as compared to parental HEK293 cells (*filled peak*). Expression was detected with the FITC conjugated 3F10 anti-HA tag MAb. **b** Specific expression of MISIIR ECD on HEK::MISIIR"+" (*unfilled line*) vs. parental HEK293 cells (*filled peak*) as detected by an anti-MISIIR ECD monoclonal antibody [44] and a secondary FITC conjugated anti-mouse IgG. The secondary antibody failed to bind to either cell line alone (data not shown)

To validate our strategy we carried out a selection with the non-immune library described above. Empirically, the first round of traditional, solid-phase, panning typically results in an output of 10^4–10^6 cfu from a naïve library, shedding a hint on the possible numbers of antigen-specific scFv contained within the library. Poul demonstrated that typical cell-based strategies result in 1–12 phage bound per cell [40]. Based on this criteria we reasoned that a minimum of 10^4 HEK::MISIIR"+" cells would be sufficient to isolate all phage specific for the MISIIR ECD during early rounds of selection where no enrichment would be predicted. We carried out the selection through three rounds of panning with the initial round consisting of 4×10^{12} phage incubated with 2×10^4 CellTracker-stained HEK::MISIIR"+" cells in the presence of a 500-fold excess of unstained HEK::MISIIR"−" cells. FACS recovery of HEK::MISIIR"+" cells was accomplished with a 70% efficiency and resulted in the isolation of 1.4×10^4 cfu. Despite variations in both the absolute ratio of antigen positive to antigen negative cells and the washing stringency used during the three rounds of panning, the HEK::MISIIR"+" cells were isolated with similar efficiencies. In addition, a similar phage to HEK::MISIIR"+" cell ratio was obtained in each of the three rounds of selection. Rounds 1 and 2 provided average of 1 phage/cell and round 3 provided an average of 2 phage/cell (Table 2).

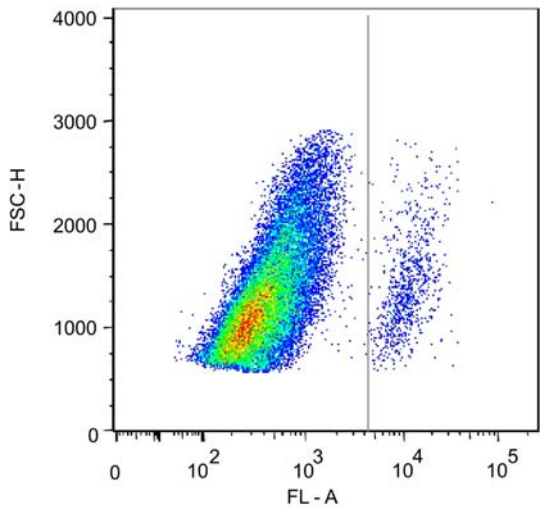

Fig. 2 Flow cytometry-based separation of vital dye stained HEK::MISIIR"+" cells from unstained HEK::MISIIR"−" cells. HEK::MISIIR"+" cells uniformly stained with the vital dye CellTracker Green were added to unstained HEK::MISIIR"−" cells at a final concentration of 0.02%. Stained cells (*right*) were readily separated from unstained cells (*left*) by flow cytometry

Table 2 Cell sorter-based biopanning selection of MISIIR-specific scFv phage clones

Sorting round	HEK::MISIIR"−" ($\times 10^6$)	HEK::MISIIR"+" ($\times 10^6$)	% of input[a]	# Washes	Recovered cells	% Recovery	Colony number[b]	Positive rate[c]
1st	10	0.02	0.2	1	14,270	70	1.4×10^4	24/96
2nd	15	0.02	0.13	5	10,030	50	1.0×10^4	26/96
3rd	20	0.2	1	1	153,374	75	3×10^5	22/96

Three rounds of cell-sorter-based selection were performed to isolate scFv phage clones that bound preferentially to HEK::MISIIR"+" cells. In each round, a large excess of HEK::MISIIR"−" cells were preincubated with the scFv phage clones to absorb the majority of phage-antibodies that were specific for normal HEK cell surface antigens. Cell Tracker Green (Invitrogen) labeled target cells (HEK::MISIIR"+") were added 1 h later and the mixture was incubated with rotation for 2 h at room temperature. The cells were washed as indicated and Cell Tracker Green labeled target cells with scFv phage bound to their surface were isolated by cell sorting. Monophage ELISA was used to determine positive binding clones

[a] Percent HEK::MISIIR"+" Cell Tracker stained cells in the selection

[b] Based upon titer of independent transformants after recovered phage infection of TG1 *E. coli*

[c] As determined by monophage ELISA

Characterization of MISIIR-specific scFvs

Specificity of the isolated scFvs for MISIIR was initially evaluated by monophage ELISA against purified, recombinant, MISIIR ECD:Fc [54]. In stark contrast to what is typically seen in solid phase panning strategies, 24 of the initial 96 clones analyzed from the first round displayed ELISA values that were three- to tenfold above background, indicative of rapid identification of antigen-specific scFv-phage by this strategy. However, the percentage of randomly selected phage (96/round) that were positive for the MISIIR ECD:Fc when tested in the ELISA format did not increase from round 1 to round 3. In all three rounds 23–27% of the tested clones displayed specific binding to the MISIIR ECD. Consistent with the apparent lack of amplification of the MISIIR-specific phage from round 1 to round 3, the diversity of the positive clones decreased in successive rounds. PCR-based fingerprint analysis of clones isolated in the first round of panning identified seven unique patterns with sequencing analysis confirming the presence of four unique, full-length, scFvs (GY1, 4, 5, and 7) with the remaining three patterns encoding truncated scFvs that were removed from further evaluation. GY1, 4, and 7 were identified in round 2 and GY1 and GY4 were identified in round 3. This trend is indicative of selective enrichment of the MISIIR-specific phage in successive rounds of panning.

Surface plasmon resonance (SPR) with purified antibodies was used to confirm the binding specificity implied by the monophage ELISA results. Consistent with the ELISA results, purified GY1 and GY7 scFvs bound specifically to the ECD of MISIIR (Fig. 3). The GY4 scFv proved to be unstable when expressed as an isolated protein in either bacteria or transient transfection of COS cells (data not shown). However, when expressed as an scFv:Fc in a manner similar to what was described previously [54], the GY4 scFv demonstrated specific binding to the MISIIR ECD

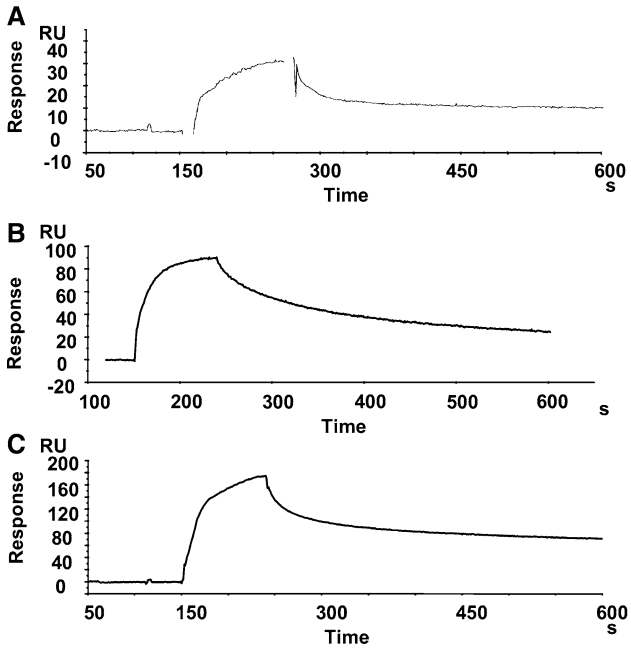

Fig. 3 SPR analysis of anti-MISIIR antibodies isolated by cell sorter selections. The anti-MISIIR antibodies were expressed and purified and the ability the GY1 scFv (**a**), GY7 scFv (**b**) and GY4 scFv:Fc (**c**) to bind to recombinant MISIIR ECD:Fc immobilized on a CM5 sensor chip was determined on a BIAcore 1000

(Fig. 3). In contrast to the ELISA results, the GY5 scFv identified during the first round of screening, failed to bind to the MISIIR ECD by SPR, providing a possible explanation for its loss in subsequent rounds of biopanning. As further proof of specificity, when converted into dimeric scFv:Fc constructs GY1 (data not shown) and GY4 (Fig. 4) bound specifically to two MISIIR-positive cell lines, AN3CA [42] and HEK::MISIIR"+", when analyzed by flow cytometry. In addition, dimeric forms of GY1 and GY7 scFvs (formed by cross-linking the scFvs via a MAb specific for the 6×HIS affinity tag on the C-terminus of the proteins)

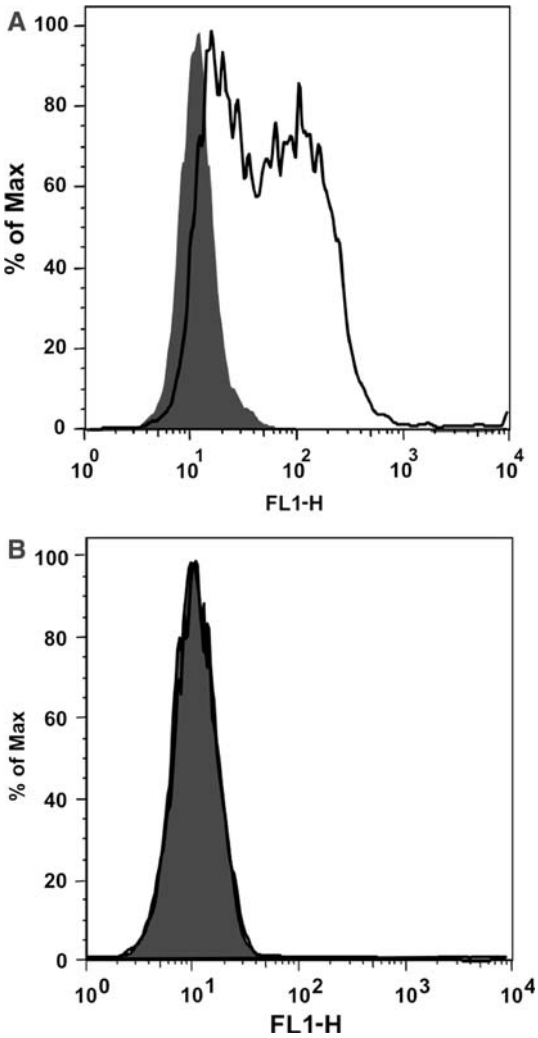

Fig. 4 Flow cytometric analysis of the anti-MISIIR GY4 scFv:Fc molecule. The ability of the GY4 scFv:Fc to bind to the MISIIR positive AN3Ca endometrial carcinoma cell line (**a**) and the MISIIR negative COS cell line was evaluated by flow cytometry. GY4 scFv:Fc bound to MISIIR positive AN3Ca cells and failed to bind to MISIIR negative COS cells. Secondary antibody controls are presented as *solid dark peaks* and the shift associated with the binding of the GY4 scFv:Fc is indicated by the *unfilled lines*

also bound specifically by flow cytometry, albeit with lower efficiency than the scFv:Fc constructs (data not shown).

GY-4 scFv:Fc can function as an MISIIR-directed immunotoxin

The use of immunotoxins to deliver catalytic toxins, such as saporin and ricin, to antigen-positive cancer cells is an effective strategy for killing cancer cells in vitro and has shown promising results in clinical settings [39]. To test the hypothesis that MISIIR can serve as a target for an immunotoxin-based therapy strategy we took advantage of the commercially available anti-human IgG-saporin immunoconjugate, Hum-ZAP. When treated with doses of GY4 scFv:Fc ranging from 1 to 100 nM, in combination with a constant concentration (50 ng) of Hum-ZAP, the complex displayed a dose-independent cytotoxicity when normalized to the results obtained by treating AN3CA cells with Hum-ZAP alone (Fig. 5a). Treatment with an equal dose of Hum-ZAP alone had no effect on cell survival over mock treated cells. The level of cytotoxicity elicited by GY4/Fc and Hum-ZAP was significantly greater ($P \leq 0.05$) than that elicited by trastuzumab and Hum-ZAP at all dose levels tested ($P \leq 0.04$). Cytotoxicity seen at the 1 and 10 nM doses of GY4/Fc and Hum-ZAP was also significantly greater than that elicited by GY4/Fc alone ($P \leq 0.02$). Although the cytotoxicity seen with 100 nM GY4/Fc and Hum-ZAP was not significantly different than that seen at the other dose levels with this combination ($P \geq 0.6$), it failed to meet the criteria for statistical significance in a direct comparison with 100 nM GY4/Fc alone ($P = 0.055$) due in a large part to the greater standard deviation seen at this dose. Consistent with the GY4/Fc and Hum ZAP complex resulting in an MISIIR-dependent cytotoxicity the complex failed to elicit a cytotoxic response against MISIIR-negative COS7 cells. Comparisons between equal doses of GY4/Fc and Hum-ZAP and GY4/Fc show similar levels of growth (Fig. 5b, $P > 0.05$). AN3CA cells express high levels of EGFR but little to no HER2 (data not shown). Consistent with this expression pattern, treatment of AN3CA cells with a complex comprised of the anti-EGFR MAb ABX-EGF and Hum-ZAP, at 1 and 10 nM concentrations of ABX-EGF, inhibits growth as compared to treatment with equal doses of a complex comprised of the anti-HER2 MAb trastuzumab and Hum-ZAP (Fig. 5a, $P < 0.001$). Interestingly, the observed cytotoxicity of ABX-EGF and Hum-ZAP is inversely proportional to the administered dose, with the 100 nM dose of ABX-EGF failing to elicit a statistically significant level of cytotoxicity as compared to the trastuzumab control ($P = 0.08$). Intriguingly, treatment of COS7 cells with ABX-EGF and Hum-ZAP results in a similar pattern of killing, albeit to a lesser extent, as was seen with AN3CA. This suggests cross-reactivity between the monkey EGFR and the ABX-EGF antibody. In contrast, no killing is seen with trastuzumab and Hum-ZAP despite the expression of the HER2 ortholog on COS7 cells. This is entirely consistent with the known specificity of trastuzumab for human HER2. Experiments are currently underway to establish the ability of GY4/Fc to function as an immunotoxin, both in vitro and in vivo, when directly conjugated to saporin.

Discussion

A number of technologies such as phage display [20, 35, 53] and ribosome display [21] as well as the emergence of

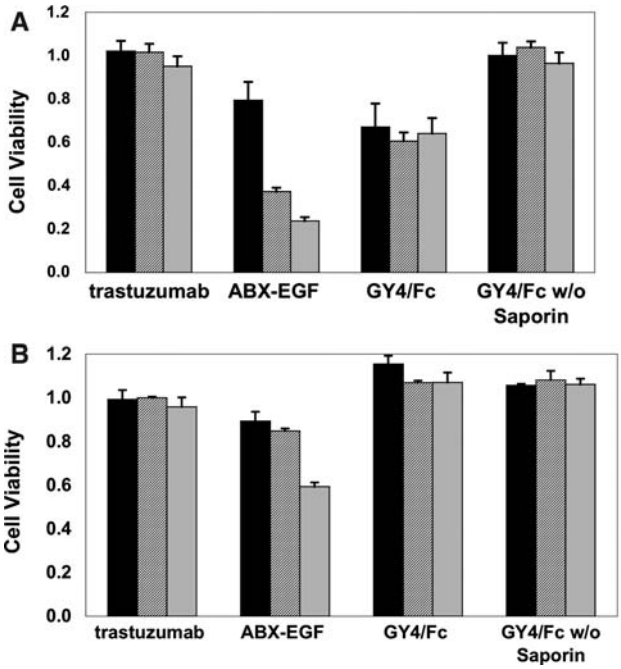

Fig. 5 Anti-MISIIR GY4 scFv:Fc molecules are capable of directing saporin-immunoconjugates to kill MISIIR expressing tumor cells. The ability of the anti-MISIIR GY4 scFv:Fc to target cytotoxic agents selectively to MISIIR expressing cells was determined using the commercially available anti-human IgG-saporin immunoconjugate, Hum-ZAP. 100 nM (*black*), 10 nM (*hatched*) or 1 nM (*gray*) of trastuzmab, ABX-EGF or GY4 scFv:Fc were added to MISIIR expressing AN3Ca cells (**a**) or MISIIR negative COS cells (**b**) that were preincubated with 50 ng/well of anti-human IgG-saporin immunoconjugate. GY4 scFv:Fc was added to the cells without anti-human IgG-saporin immunoconjugate (*far right group of each graph*) indicated that the GY4 scFv:Fc molecules did not elicit cytotoxic effects without the addition of the saporin immunoconjugate. *Bars* represent cell viability expressed as a ratio of viability after treatment with antibody/Hum-ZAP combinations to viability associated with anti-human IgG-saporin immunoconjugate treatment alone; no effect of a combination on cell viability = 1.0. *Lines* indicate standard deviation

alternate antibody-like scaffolds [47] have revolutionized the ability of scientists to isolate novel proteins that are reactive with tumor associated self-antigens that previously were difficult to target. However, the techniques employed to identify and isolate the desired binding proteins are often suboptimal. Immobilization of proteins on plastic media for phage panning procedures leads to conformational changes of the target protein [5, 6] that can favor the isolation of antibodies that are unable, or reduced in their ability, to bind to the native protein [11]. Solution-based selection strategies using biotinylated target protein and streptavidin-conjugated magnetic beads often results in a predominance of anti-streptavidin antibodies or the isolation of clones that bind to sites created by the biotinylation procedure or the target/biotin/streptavidin complex (M. K. Robinson, unpublished data), each of which will ultimately fail to bind to relevant antigen expressed on the surface of target cells. Ideally, selections of antibodies would be performed using proteins expressed in their native conformation on the surface of target cells. However, this approach typically leads to the isolation of a variety of antibodies with specificities for other cell surface proteins, not just those that are associated with the tumor phenotype [40]. The difficulties associated with cell-based selections are further confounded when a target antigen is expressed in low abundance as is the case with MISIIR.

MISIIR is potentially a very promising target for antibody-based treatment of ovarian cancer. Functional MISIIR is expressed in a restricted manner on the mesenchymal cells surrounding the Müllerian ducts and follicular structures of fetal gonads during embryogenesis [3]. Its potential utility as a target in cancer therapy derives from its broad expression on granulosa cell tumors [25], primary ovarian tumors, ovarian cancer cells isolated from ascities and banked ovarian cancer cell lines [33]. Ovarian cancer cell lines and primary ovarian cancer cells isolated from ascities in patients undergo apoptosis following treatment with MIS [9, 15, 16, 18, 33, 48]. Recombinant human MIS has also been found to inhibit the growth of ovarian cancer xenografts growing in immunodeficient mice [48]. Taken together, these studies underscore the potential utility of MISIIR as a therapeutic membrane target. Anti-MISIIR antibodies that mimic MIS and trigger signaling through the receptor could emerge as potent naked immunotherapeutic agents while those that selectively target the receptor without initiating signaling events could be employed to deliver cytotoxic agents to tumors in a highly specific manner. However, there are only two reports describing the production of monoclonal antibodies or engineered antibody fragments that are specific for MISIIR [44, 54]. This is likely due to the difficulties encountered in expressing MISIIR ECD and its highly conserved nature between species. Salhi et al. [44] addressed these issues by immunizing mice with human MISIIR ECD expressed in *E. coli* and succeeded in generating one MAb (12G4) that has proven to be effective for IHC applications but does not target MISIIR in vivo. More recently, we have reported the successful creation of two fusion proteins composed of human MISIIR ECD and human IgG Fc domains with the MISIIR ECD located at either the carboxy or amino terminus [54]. These fusion proteins were employed in the isolation of MISIIR-specific scFv molecules from a naïve human scFv phage display library. However, we found that the use of a fusion protein as a target led to the isolation of numerous clones that were only capable of binding to MISIIR ECD in one orientation (e.g., 5′ or 3′ of the Fc domain). This suggested to us that a majority of the clones we isolated were specific for the fusion junction or for sequences that were sterically obscured by the presence of the Fc domain. A final limitation of the anti-MISIIR antibodies described by both Yuan et al.

and Salhi et al. was their impaired utility due to relatively weak affinity constants.

Our initial step in the current report was to generate a very large combinatorial human naïve scFv phage display library to provide a robust source of anti-MISIIR scFv clones. In constructing our library we employed a number of steps to increase its size and diversity. First, non-immune human B-cells cells were isolated from a large number of donors from diverse ethnic origins and employed as a source of RNA extraction. Second, nested-PCR using forward primers (derived from the constant region) and backward outside primers derived from the leader-coding regions of antibodies were employed to comprehensively amplify variable domain antibody segments. To the best of our knowledge, this represented the first time that nested PCR was employed to amplify human antibody fragments. Third, a two-step cloning strategy was employed in place of the more common "One pot" method in order to increase the rate of correct recombination of scFv genes [31]. Analysis of the final naïve scFv library revealed a high percentage of intact scFv, suggesting potential advantages of two-step cloning strategies. Furthermore, a sampling of randomly picked clones demonstrated that diversity was maintained in the final library estimated to contain 2.3×10^{10} unique clones.

While cell-based panning is a powerful technique for the isolation of antibodies that recognize cell surface proteins, the previously reported methods failed to discriminate between the desired tumor-associated antigens and "normal" cell-surface proteins that are widely expressed on normal tissues. de Kruif et al. [13] first reported on the use of cell sorting to isolate desired subpopulations of specific antibodies from phage libraries. Specific antibodies were retrieved from a semi-synthetic human germline antibody library employing fluorescence conjugated anti-CD3 and/or anti-CD20 antibody labeled cells. However, the number (millions) of target cells they used was significantly greater than the tens of thousands of cells we used in the study described here. Other reports of cell-based panning describe the use of several million target cells in combination with a few fold excess background (absorber) cells and/or the use of immunized libraries to increase the quantity of phage antibodies that are capable of binding to the tumor-associated antigens [7, 24]. One group, Poul et al. [40], reported utilizing cell-based selection strategies to isolate antibodies that specifically targeted internalizable antigens. Although the procedure did result in the isolation of a number of interesting scFv molecules, the large majority bound to common cell-surface proteins (e.g. transferrin). The combination of solid-phase panning and cell-based panning can be more efficient than either method alone in isolation of specific antibodies to native targets [52]. Nevertheless, these results reflected the difficulty in identifying appropriate negative control cell lines that express all the normal "housekeeping" proteins but lack the desired target antigen for use in a pre-panning library depletion step. This is particularly problematic when the target antigen is expressed in low abundance on the cell surface.

For the selections described here, aliquots of our naïve human scFv phage display library were mixed with a large number (10–20 million) of HEK::MISIIR"−" (empty vector transfected) cells to facilitate the adsorption of the scFv-phage clones that were specific for other proteins naturally present on the cells. A significantly smaller number (0.02–0.2 million) of HEK::MISIIR"+" (CellTracker Dye-stained transfected) cells expressing the MISIIR ECD target antigen were then added to the mixture. By employing stained cells, those expressing the desired target antigen and the bound scFv-phage particles could be separated from the HEK::MISIIR"−" cells on a cell sorter. This allowed us to eliminate a depletion step, thereby decreasing the likelihood that desired scFv-phage clones would be lost during a washing step. After the completion of three rounds of selection, four unique anti-MISIIR ECD scFv clones were isolated. All four were expressed in *E. coli* and three exhibited positive binding to recombinant MISIIR ECD:Fc when evaluated by SPR on the BIAcore. The affinity of these scFv molecules for MISIIR ECD was measured and all three were observed to be in the 1 μM–10^2 nM range. Low to moderate affinity of isolated clones has been a common issue with cell-based selections that can sometimes be resolved by combining less stringency in the early rounds of selection with greater stringency in the later rounds. However, our previous attempts to isolate anti-MISIIR scFv clones from a different naïve human scFv phage display library using common solid phase selection strategies also resulted in a low number of unique clones, all of which had low to moderate affinity [54], suggesting that this may be related to the antigen as well.

When evaluated in flow cytometry studies, all three scFvs exhibited at best minimal binding to MISIIR ECD expressing cells and AN3Ca cells. This was consistent with our past observations that it is difficult to evaluate binding specificity in this assay with monovalent scFv molecules with affinities in this range. Bivalent molecules can be readily formed using antibodies directed against the FLAG [17] and Myc [50] tags. Using this strategy, we were able to detect significant binding of all three scFv molecules by flow cytometry when we preincubated them with MAbs specific for their c-terminal 6× His tags prior to adding them to the cells. This provided us with both evidence of binding to cell-based MISIIR and the impetus for engineering the scFv clones into divalent scFv/Fc molecules.

Due to its higher avidity the GY4/Fc was selected for preliminary evaluation as a vehicle for the delivery of cyto-

toxic agents for the treatment of cancer. In in vitro assays, GY4/Fc was capable of targeting, internalizing a secondary anti-human IgG Fc-saporin immunoconjugate (Hum-ZAP) and killing MISIIR expressing AN3Ca cells but not MISIIR negative COS cells. An interesting observation in the assay was that lower concentrations of primary antibodies were more effective at cell killing. Although true for both GY4/Fc and ABX-EGF the effect was enhanced when cells were treated with ABX-EGF. A possible explanation for the decreased cytotoxicity observed at higher antibody doses is the potential to favor monovalent association due to antigen saturation. This in turn would be hypothesized to lower the rate of internalization and degradation of the immunotoxin complex. Alternatively, the ability of primary antibodies to bind cell surface receptors may be inhibited by incorporation into a complex with Hum-ZAP. The Hum-ZAP is predicted to be saturated even at the lowest antibody:Hum-ZAP ratio (range from 4:1 to 400:1) leaving excess free primary antibody to compete with the antibody/Hum-ZAP complex for the cell surface receptors, leading to decreased concentrations of saporin transported into the cell. Primary antibodies are predicted to be in excess over their target receptors even at the lowest concentration of primary antibody. Our current efforts are focused on developing directly conjugated immunotoxin and immunodrug molecules based upon the anti-MISIIR scFv/Fc clones described here and on the isolation and identification of additional anti-MISIIR antibodies that are capable of directly killing MISIIR expressing malignant cells.

In summary, we have described a novel method for isolating antibodies directed against known antigenic targets. This approach has the advantage of presenting the antigen in a relevant, functional orientation in a natural setting. Our method was validated by the isolation of scFv molecules specific for a difficult to express tumor-associated antigen, human MISIIR. In vitro evaluation of the resulting anti-MISIIR scFv molecules provided both a functional validation of this selection strategy and the first evidence of targeted killing of tumor cells by anti-MISIIR immunoconjugates, suggesting that this selectively expressed antigen could provide a promising target for cancer therapy.

Acknowledgments The work was supported by National Cancer Institute Ovarian Specialized Programs of Research Excellence grant P50 CA83638. The authors would like to thank Dr. Isabelle Navarro-Teulon of the Centre de Recherche en Cancérologie, INSERM-Université Montpellier France for the kind gift of the 12G4 MAb, Drs. R. Kathy Alpaugh and Jonathan Cheng of the Fox Chase Cancer Center for their assistance in collecting human blood for the production of the scFv library; Dr. Yong Tang of the Fox Chase Cancer Center and Dr. Jinchao Zhang of the Beijing Institute of Biotechnology for helpful suggestions in phage library construction, James F. Oesterling of the Fox Chase Cancer Center Flow Cytometry Facility for assistance with the cell sorter, the Fox Chase Cancer Center Biotechnology Facility for oligonucleotide synthesis and the Fox Chase Cancer Center DNA Sequencing Facility for expert technical assistance.

References

1. Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD, Weiner LM (1998) Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res 58:485–490
2. Adams GP, Weiner LM (2005) Monoclonal antibody therapy of cancer. Nat Biotechnol 23:1147–1157
3. Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA (1995) Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951–4962
4. Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP, Uilenbroek JT, Karels B, Wilming LG, Meijers JH et al (1994) A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the mullerian duct. Development 120:189–197
5. Butler JE, Navarro P, Sun J (1997) Adsorption-induced antigenic changes and their significance in ELISA and immunological disorders. Immunol Invest 26:39–54
6. Butler JE, Ni L, Nessler R, Joshi KS, Suter M, Rosenberg B, Chang J, Brown WR, Cantarero LA (1992) The physical and functional behavior of capture antibodies adsorbed on polystyrene. J Immunol Methods 150:77–90
7. Cai X, Garen A (1995) Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc Natl Acad Sci USA 92:6537–6541
8. Carter P, Smith L, Ryan M (2004) Identification and validation of cell surface antigens for antibody targeting in oncology. Endocr Relat Cancer 11:659–687
9. Chin TW, Parry RL, Donahoe PK (1991) Human mullerian inhibiting substance inhibits tumor growth in vitro and in vivo. Cancer Res 51:2101–2106
10. Coomber DW (2002) Panning of antibody phage-display libraries. Standard protocols. Methods Mol Biol 178:133–145
11. Darst SA, Robertson CR, Berzofsky JA (1988) Adsorption of the protein antigen myoglobin affects the binding of conformation-specific monoclonal antibodies. Biophys J 53:533–539
12. Davis CG, Jia XC, Feng X, Haak-Frendscho M (2004) Production of human antibodies from transgenic mice. Methods Mol Biol 248:191–200
13. de Kruif J, Terstappen L, Boel E, Logtenberg T (1995) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc Natl Acad Sci USA 92:3938–3942
14. de Rock E, Taylor N (1977) An easy method of layering blood over Ficoll-Paque gradients. J Immunol Methods 17:373–374
15. Donahoe PK, Fuller AF Jr, Scully RE, Guy SR, Budzik GP (1981) Mullerian inhibiting substance inhibits growth of a human ovarian cancer in nude mice. Ann Surg 194:472–480
16. Donahoe PK, Swann DA, Hayashi A, Sullivan MD (1979) Mullerian duct regression in the embryo correlated with cytotoxic activity against human ovarian cancer. Science 205:913–915
17. Ellmark P, Esteban O, Furebring C, Malmborg Hager AC, Ohlin M (2002) In vitro molecular evolution of antibody genes mimicking receptor revision. Mol Immunol 39:349–356
18. Fuller AF Jr, Budzik GP, Krane IM, Donahoe PK (1984) Mullerian inhibiting substance inhibition of a human endometrial carcinoma cell line xenografted in nude mice. Gynecol Oncol 17:124–132
19. Gould LH, Sui J, Foellmer H, Oliphant T, Wang T, Ledizet M, Murakami A, Noonan K, Lambeth C, Kar K, Anderson JF, de Silva AM, Diamond MS, Koski RA, Marasco WA, Fikrig E (2005)

Protective and therapeutic capacity of human single-chain Fv-Fc fusion proteins against West Nile virus. J Virol 79:14606–14613
20. Griffiths AD, Malmqvist M, Marks JD, Bye JM, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC et al (1993) Human anti-self antibodies with high specificity from phage display libraries. EMBO J 12:725–734
21. Hanes J, Pluckthun A (1997) In vitro selection and evolution of functional proteins by using ribosome display. Proc Natl Acad Sci USA 94:4937–4942
22. He WW, Gustafson ML, Hirobe S, Donahoe PK (1993) Developmental expression of four novel serine/threonine kinase receptors homologous to the activin/transforming growth factor-beta type II receptor family. Dev Dyn 196:133–142
23. Heitner T, Moor A, Garrison JL, Marks C, Hasan T, Marks JD (2001) Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library. J Immunol Methods 248:17–30
24. Hoogenboom HR, Lutgerink JT, Pelsers MM, Rousch MJ, Coote J, Van Neer N, De Bruine A, Van Nieuwenhoven FA, Glatz JF, Arends JW (1999) Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. Eur J Biochem 260:774–784
25. Imbeaud S, Faure E, Lamarre I, Mattei MG, di Clemente N, Tizard R, Carre-Eusebe D, Belville C, Tragethon L, Tonkin C, Nelson J, McAuliffe M, Bidart JM, Lababidi A, Josso N, Cate RL, Picard JY (1995) Insensitivity to anti-mullerian hormone due to a mutation in the human anti-mullerian hormone receptor. Nat Genet 11:382–388
26. Kettleborough CA, Saldanha J, Heath VJ, Morrison CJ, Bendig MM (1991) Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation. Protein Eng 4:773–783
27. Kingsbury GA, Junghans RP (1995) Screening of phage display immunoglobulin libraries by anti-M13 ELISA and whole phage PCR. Nucleic Acids Res 23:2563–2564
28. Kohler G, Hengartner H, Shulman MJ (1978) Immunoglobulin production by lymphocyte hybridomas. Eur J Immunol 8:82–88
29. Kohls MD, Lappi DA (2000) Mab-ZAP: a tool for evaluating antibody efficacy for use in an immunotoxin. Biotechniques 28:162–165
30. Krag DN, Shukla GS, Shen GP, Pero S, Ashikaga T, Fuller S, Weaver DL, Burdette-Radoux S, Thomas C (2006) Selection of tumor-binding ligands in cancer patients with phage display libraries. Cancer Res 66:7724–7733
31. Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Pluckthun A (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Methods 201:35–55
32. Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222:581–597
33. Masiakos PT, MacLaughlin DT, Maheswaran S, Teixeira J, Fuller AF Jr, Shah PC, Kehas DJ, Kenneally MK, Dombkowski DM, Ha TU, Preffer FI, Donahoe PK (1999) Human ovarian cancer, cell lines, and primary ascites cells express the human Mullerian inhibiting substance (MIS) type II receptor, bind, and are responsive to MIS. Clin Cancer Res 5:3488–3499
34. Matson CF (1965) Polyacrylamide gel electrophoresis. A simple system using gel columns. Anal Biochem 13:294–304
35. McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554
36. Mishina Y, Tizard R, Deng JM, Pathak BG, Copeland NG, Jenkins NA, Cate RL, Behringer RR (1997) Sequence, genomic organization, and chromosomal location of the mouse Mullerian-inhibiting substance type II receptor gene. Biochem Biophys Res Commun 237:741–746
37. Nagy P, Friedlander E, Tanner M, Kapanen AI, Carraway KL, Isola J, Jovin TM (2005) Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Res 65:473–482
38. Nef S, Parada LF (2000) Hormones in male sexual development. Genes Dev 14:3075–3086
39. Pastan I, Hassan R, Fitzgerald DJ, Kreitman RJ (2006) Immunotoxin therapy of cancer. Nat Rev Cancer 6:559–565
40. Poul MA, Becerril B, Nielsen UB, Morisson P, Marks JD (2000) Selection of tumor-specific internalizing human antibodies from phage libraries. J Mol Biol 301:1149–1161
41. Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E (1998) Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. J Clin Invest 102:430–437
42. Renaud EJ, MacLaughlin DT, Oliva E, Rueda BR, Donahoe PK (2005) Endometrial cancer is a receptor-mediated target for Mullerian Inhibiting Substance. Proc Natl Acad Sci USA 102:111–116
43. Ross JS, Schenkein DP, Pietrusko R, Rolfe M, Linette GP, Stec J, Stagliano NE, Ginsburg GS, Symmans WF, Pusztai L, Hortobagyi GN (2004) Targeted therapies for cancer 2004. Am J Clin Pathol 122:598–609
44. Salhi I, Cambon-Roques S, Lamarre I, Laune D, Molina F, Pugniere M, Pourquier D, Gutowski M, Picard JY, Xavier F, Pelegrin A, Navarro-Teulon I (2004) The anti-Mullerian hormone type II receptor: insights into the binding domains recognized by a monoclonal antibody and the natural ligand. Biochem J 379:785–793
45. Schier R, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman MA, Huston JS, Houston LL, Weiner LM, Adams GP (1995) In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1:73–81
46. Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindquist E, Schier R, Hemingsen G, Wong C, Gerhart JC, Marks JD (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. Proc Natl Acad Sci USA 95:6157–6162
47. Skerra A (2000) Engineered protein scaffolds for molecular recognition. J Mol Recognit 13:167–187
48. Stephen AE, Pearsall LA, Christian BP, Donahoe PK, Vacanti JP, MacLaughlin DT (2002) Highly purified mullerian inhibiting substance inhibits human ovarian cancer in vivo. Clin Cancer Res 8:2640–2646
49. Teixeira J, He WW, Shah PC, Morikawa N, Lee MM, Catlin EA, Hudson PL, Wing J, Maclaughlin DT, Donahoe PK (1996) Developmental expression of a candidate mullerian inhibiting substance type II receptor. Endocrinology 137:160–165
50. Wang X, Campoli M, Ko E, Luo W, Ferrone S (2004) Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies. J Immunol Methods 294:23–35
51. Watters JM, Telleman P, Junghans RP (1997) An optimized method for cell-based phage display panning. Immunotechnology 3:21–29
52. Williams B, Atkins A, Zhang H, Lu D, Jimenez X, Li H, Wang MN, Ludwig D, Balderes P, Witte L, Li Y, Zhu Z (2005) Cell-based selection of internalizing fully human antagonistic antibodies directed against FLT3 for suppression of leukemia cell growth. Leukemia 19:1432–1438
53. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. Annu Rev Immunol 12:433–455
54. Yuan QA, Simmons HH, Robinson MK, Russeva M, Marasco WA, Adams GP (2006) Development of engineered antibodies specific for the Mullerian inhibiting substance type II receptor: a promising candidate for targeted therapy of ovarian cancer. Mol Cancer Ther 5:2096–2105