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Recombinant single-chain variable fragment antibodies against extracellular epitopes of human multidrug resistance protein MRP3 for targeting malignant gliomas

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Multidrug resistance protein 3 (MRP3), a multidrug resistance protein identified by serial analysis of gene expression as a glioblastoma multiforme (GBM)-associated molecule, is highly expressed in GBM, but not in normal brain cells. Thus, MRP3 is a candidate for GBM immunotargeting, but to date, no monoclonal antibody has been isolated that can target an extracellular MRP3 epitope. By phage display, we have isolated 3 recombinant, fully human, single-chain Fv (scFv) antibodies, M25, M58 and M89, which specifically react with the extracellular N-terminus of human MRP3. In ELISA, these scFvs reacted only with the peptide used for screening and not with other MRP3-derived peptides. Flow cytometric analysis revealed that these scFv fragments bind specifically to viable human GBM cells displaying different MRP3 expression levels, but not to MRP3-null cells. Furthermore, these scFv antibodies failed to react with tumor cells overexpressing other MRP proteins, including MRP1, MRP2, MRP4 and MRP5. M25 and M58 also bound to viable neurospheres. Iodogen-labeled scFvs demonstrated a yield of 56–76%. The immunoreactive fractions of the radiolabeled M25, M58 and M89 scFvs were 32, 52 and 69%, respectively. M25 exhibited 20% internalization into D2159MG neurospheres, M58, 33% into D54MG cells and M89, 26% into D247MG. Immunohistochemical evaluation of human gliomas to determine the localization of MRP3 antigen using scFvs M25 and M58 showed a dense cytoplasmic and membranous staining pattern. These Fv-based recombinant antibodies, which possess superior tumor penetration capabilities and selectively target tumor cells that express MRP3, may potentially be used in immunotherapy and diagnosis for brain tumors and other cancers.

Human multidrug resistance protein 3 (MRP3), also known as cMOAT2 or ABCC3, is an organic anion and multidrug extruding transporter. It confers multidrug resistance on human cancer cells by decreasing the intracellular concentration of drugs, which results in cancer treatment failure.[1] Along with several other bacterial and eukaryotic transporters, MRP3 is a member of the C-branch of the ATP binding cassette (ABC transporter).[2] The human MRP gene family has multiple members (MRP1–MRP9),[3,4] among which MRP3 has a molecular weight of 190–200 kDa and is closest in structure to MRP1, with 58% amino acid identity.[2] Functional analysis has revealed that MRP3 is involved in the cellular extrusion of organic anions[5] and can transport monovalent bile acids (as taurocholate and glycocholate).[6]

Key words: GBM, MRP3, scFv antibody, phage display

Abbreviations: ABC: ATP-binding cassette; BSA: bovine serum albumin; FITC: fluorescein isothiocyanate; GBM: glioblastoma multiforme; HBSS: HEPES balanced salt solution; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HGG: high-grade glioma; HRP: horseradish peroxidase; K_A: affinity at binding equilibrium; MRP: multidrug resistance protein; SAGE: serial analysis of gene expression; scFv: single-chain, variable fragment antibody; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMA: tissue microarray

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Glucuronate conjugates are preferred substrates for MRP3, whereas nonconjugated organic anions (such as methotrexate), bile acid sulfates (such as taurolithocholate sulfate) and glutathione conjugates are poor substrates for MRP3.[5,6] In normal human tissues, MRP3 is expressed mainly in the liver, adrenal gland, placenta, testis, intestine, colon and gallbladder and at a relatively lower level in pancreas, kidney, lung and tonsils.[7] Hepatic expression of MRP3 has been linked to the concentration of serum bilirubin or its glucuronides, which suggests that these endogenous compounds may be involved in the induction of MRP3.[8] Expression of MRP3 has also been reported in some human cell lines, including Caco-2 and HepG2,[9] and overexpression of MRP3 has been observed in some tumor cell lines that have acquired multidrug resistance.[10,11] MRP3 has also been reported to play a role in progression of adult acute myeloid leukemia.[12]

It has recently been shown that hepatic progenitor cells have high expression levels of functional MRP1 and MRP3, which may have a role in removing either exogenous or endogenous toxins and metabolites from progenitor cells.[13] Bronger *et al.* have also detected the expression of other MRP family members such as MRP4 and MRP5 in the blood–brain barrier and in glioma cells at the protein level.[14] Localization of MRP1, MRP4 and MRP5 protein in rapidly frozen perilesional samples of several regions of adult human brain was reported by Nies *et al.*[15] Expression of ABC transporters was also found to be responsible for the highly enriched side population phenotype in a wide variety of stem cells,[16] and other initial studies also showed that they may be active in hematopoietic stem cells as functional regulators.[17] Calatozzolo *et al.* showed that *MRP3* is hyperexpressed in astrocytomas as the primary resistance to chemotherapy with drugs such as cis-platinum and carmustine[18] and that MRP3 can modulate drug sensitivity to certain anticancer agents such as cisplatin, vincristine and etoposide in human gliomas.[19] Recent research has shown that MRP3 was much less expressed in cancer stem cells. However, after differentiation, the expression of MRP3 notably rose, which suggests that just after differentiation the cells acquired chemotherapeutic resistance *via* MRP3.[20]

Glioblastoma multiforme (GBM) is the most aggressive tumor type among the astrocytic tumors. Despite recent advances and discoveries in cancer treatment, the prognosis for patients with malignant glioma is very poor. Overexpressed MRP3 protein has been implicated as one of the cell surface targets for GBM by serial analysis of gene expression (SAGE).[21] MRP3 RNA transcripts are highly expressed in GBM tissues when compared with normal brain tissues.[21]

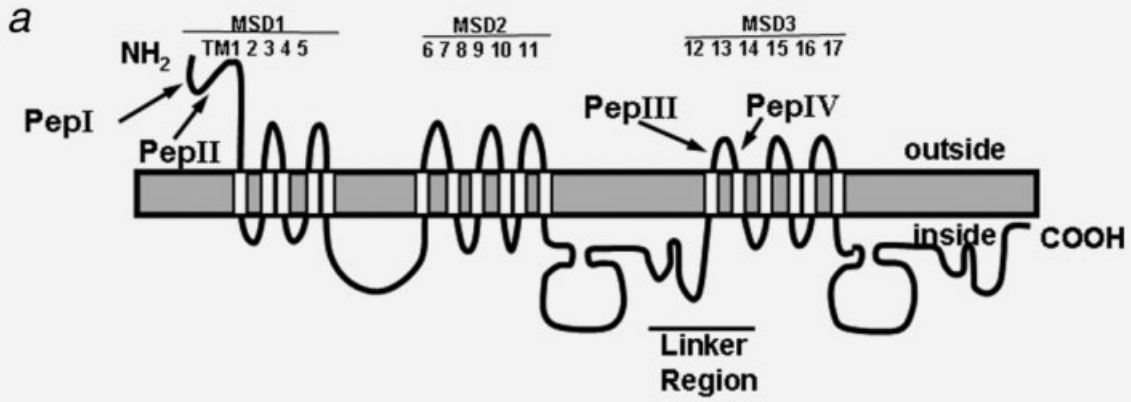
Overexpression of MRP3 in GBM and relative lack of expression in normal brain, along with its localization to the cell membrane, make MRP3 an excellent potential target for immune-based targeted therapy. Monoclonal antibodies detecting internal MRP3 epitopes have been developed, but none has been reported that detect external MRP3 epitopes. However, Fv fragments, the smallest entities that consistently maintain the binding specificity of intact antibody, have gained much interest over the past decade and are being used in a wide range of therapeutic applications, particularly in the targeted therapy against malignant cells. The Fv targeting moiety of the antibody is composed of the heavy- and light-chain variable regions (VH and VL), which are held together by a peptide linker, thereby forming a single-chain Fv molecule (scFv). Furthermore, because of recent advances in phage display technology, we can now produce scFvs with higher affinity, which circumvents the requirement for an immunization procedure.[22,23] ScFv antibodies have several advantages over conventional antibodies because they are very small. Their smaller size allows them to penetrate into tumor more efficiently and homogenously[24–27] and facilitates faster systemic and normal tissue clearance.[28,29] ScFv fragments are also finding use as tumor imaging agents and for radioimmunotherapy. ScFv molecules are being developed against a wide spectrum of cancer-specific targets and are being tested in various clinical trials, some of which already present promising anticancer activity.[30,31]

To date, no monoclonal antibody has been isolated that can target an extracellular MRP3 epitope. Here, we report, by use of a phage display approach, the first successful isolation of 3 fully human recombinant scFv antibodies, M25, M58 and M89, which specifically react with the extracellular N-terminus of the human MRP3. The scFvs reacted only with the peptide used for the phage display screening and did not crossreact with other MRP3-derived peptides. We demonstrate herein that the scFv antibody can bind to viable tumor cells with MRP3 expression but not MRP3-null cells. We show that novel recombinant antibody fragments targeting MRP3 may potentially be used for the development of cancer targeting and imaging agents for brain tumors and other cancers expressing MRP3.

Material and Methods

Cell lines

Human GBM and ependymoma-derived cell lines D247MG, D2159MG, D612EP and D633EP were established and maintained in our laboratory,[32,33] and 6B-47MG was kindly provided by Dr. David James (University of California, San Francisco). Glioma cell lines were cultured either in zinc option (ZO) medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; ZO-10% FCS) or in knockout DMEM/F-12 low-osmolality neural stem cell medium (NSCM) (Gibco) with supplements. Human ovarian carcinoma 2008 cells and their stable 2008/MRP1–3 and 2008/MRP5 transfectants with different MRP cDNA (MRP1, MRP2, MRP3 and MRP5), as well as the human embryonic kidney MRP4 transfectant cells HEK293/4.63, were kindly provided by Professor P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). All cells were incubated at 37°C in 5% CO_2 and 99% humidity.



b

MRP3-derived Peptides	Amino Acid Sequence	Amino Aacid Position	Transporter Domain
PepI	MDALCGSGELGSKFWDSNLS	1-20	N-Terminus
PepII	VHTENPDLTPGF	21-32	N-Terminus
PepIII	DAMADSRQNNTSLRL	999-1013	Extracellular Loop 6
PepIV	KIRSPQSFFDTTPSGRI	1053-1069	Extracellular Loop 6

Figure 1. Proposed membrane topology of MRP3 and location of peptides used for preparation of scFv antibodies. (*a*) Arrows indicate locations of selected peptides in the extracellular N-terminus and the extracellular loop of membrane spanning domain 3 (MSD3) of the MRP3 protein. (*b*) The amino acid sequence and position for MRP3-derived peptides used for panning of the phage display library to screen for anti-MRP3 scFv antibodies.

Table 1. Screening of human scFv naïve phage display library on MRP3-derived peptides[1]

Panning condition					PepI screening		PepII screening	
Round	Input	Type of resin	Peptide conc. (nM)	Binding time (hr)	Output (cfu)	Enrichment	Output (cfu)	Enrichment
1	1×10^{12}	Streptavidin (200 μl)	500	3	6×10^{5}	1	1×10^{5}	1
2	1×10^{12}	Avidin (40 μl)	100	1.5	5.9×10^{6}	9.8	4.4×10^{6}	44
3	1×10^{12}	Streptavidin (100 μl)	20	1	4.08×10^{7}	68	3.9×10^{7}	390

[1]Phage input and output was determined by titration, determining phage cfu (colony-forming unit) of infected *E. coli* TG1 cells before and after each round of selection.

Isolation of cells from xenografts

Human glioma and ependymoma xenografts from athymic mice were dissociated with Liberase (Roche, Indianapolis, IN) at a 100-μg/ml concentration. The dissociated cells were filtered through a disposable sieve, and reaction was inhibited with ovomucoid solution. After washing several times with media, the cells were counted and plated in either ZO-10% FCS or NSCM to isolate neurospheres. The cells were used for FACS and other analyses.

Screening of human naïve scFv phage display library

A human naïve single-chain Fv phage library (Mehta I) containing 15×10^{9} diversity was used for the selection of anti-MRP3 scFvs.[34,35] The library was subjected to 3 successive rounds of biopanning with biotinylated peptides (Anaspec, San Jose, CA) derived from MRP3 protein (PepI, PepII, PepIII and PepIV, as described and shown in Fig. 1), alternatively with streptavidin-coated beads (Dynal, Invitrogen, Carlsbad, CA) and avidin resin (Promega, Madison, WI). To eliminate phages that bind to streptavidin beads, avidin resin was used in the next round of panning followed by streptavidin beads in the third and final round. Successive reductions in the concentration of the biotinylated peptides were used: 500 nM for the first round, 100 nM for the second and 20 nM for the third round (Table 1). Streptavidin beads were mixed, and the mixture was incubated in a precoated (5% milk-phosphate-buffered saline [PBS]) tube with continuous rotation. A magnetic force was applied to separate the phages bound to the biotinylated complex. Bound phages were eluted by acidic (200 μl of 0.1 M HCl-glycine, pH 2.0) and alkaline (200 μl of 100 mM triethylamine) treatment. Neutralization was done by adding 400 μl of 1 M Tris HCl. Eluted mixtures were pooled and used to infect the amber-

codon-suppressing *E. coli* TG1 cells (A_{600}, 0.5) for 30 min at 37°C, which were later superinfected with VCSM13 interference-resistant helper phage (Stratagene, La Jolla, CA) as described by Amersdorfer and Marks.[36] More phages were grown and subjected to further rounds of selection for the enrichment of putatively positive clones for the respective peptides used for screening. The ratio of phage output to phage input was used to determine the enrichment value (Table 1).

Polyclonal and monoclonal phage antibody ELISA

The phage population produced after each round of selection was screened by ELISA for binding to MRP3 peptides and was used to confirm the round-by-round enrichment of antigen-specific phages. Phages from single, infected bacterial colonies were then screened by ELISA to identify the monoclonal phage. For polyclonal phage ELISA, aliquots of phages after each round of selection were added to 96-well plates coated with 10 μg/ml of MRP3-derived peptides conjugated with bovine serum albumin (BSA) in 100 μl of 200 mM $NaHCO_3$, pH 9.6. Plates were incubated overnight for coating with a specific antigen at 4°C. After washing with 0.05% Tween/PBS 3 times, the plates were blocked with 2% milk-PBS for 2 hr at room temperature. Wells were then briefly rinsed with PBS and incubated with phage supernatant ($\sim 10^{10}$ plaque-forming units per well) in 2% milk-PBS for 1 hr at room temperature. Plates were further incubated with horseradish peroxidase (HRP)-conjugated mouse anti-M13 antibody (1:5000, Amersham, Piscataway, NJ). Detection was performed by using a Sigmafast OPD *o*-phenylenediamine dihydrochloride tablet set (Sigma, St. Louis, MO). Phages from a single, infected bacterial colony, 1 from the second round and 1 from the third round of populations, were then screened by monoclonal phage ELISA. *E. coli* TG1 colonies were picked randomly and expanded, supernatant was added to 96-well plates coated with various MRP3-derived peptides, and ELISA was performed as described earlier.

DNA sequencing

Phage clones from the second and third rounds of panning were selected, and their DNA was isolated and sequenced by using an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences for positive clones were determined by primers "pelB forward" and "cmyc reverse," which hybridize the flanking sequence of the insert. Nucleotide sequences of the primers are as follows.

"pelBForward" (located right before the heavy chain): CATAATGAAATACCTATTGCCTA.

"cmycReverse" (located right after the light chain): CTTATTAGCGTTTGCCATT.

Several positive clones were picked on the basis of their ELISA titer, and nucleotide sequences for each of the MRP3-derived peptides were used for screening the phage library.

Production of soluble scFv

Phages expressing M25 and M58 scFvs were selected for the PepI region of MRP3, and phages expressing M89, M23 and M17 scFvs were selected for the PepII, PepIII and PepIV regions of the MRP3 protein, respectively. ScFv genes were cloned into expression vector pET 22 $b^{(+)}$ (Novagen, Madison, WI) to enable the expression of active and soluble scFv antibody. Briefly, the scFv-encoding fragment was excised from the phagemid by digesting it with NcoI and Not I restriction endonucleases. It was then ligated to the NcoI- and Not I-digested pET vector, which carries a hexahistidine tag for purification and detection. The scFv antibodies were expressed in *E. coli* BL21 (DE3) gold cells (Stratagene) and purified from the soluble cytoplasmic fraction by metal-ion affinity chromatography (Talon, Clontech, Palo Alto, CA). The M25, M58 and M89 scFvs were chosen for further analysis and characterization on the basis of their production yield, binding affinity and binding profile with cells expressing MRP3.

Affinity measurements

Kinetic measurements of purified recombinant scFv were determined by surface plasmon resonance using a BIAcore 3000 biosensor (BIAcore, Piscataway, NJ). The streptavidin chips were immobilized with an individual biotinylated peptide derived from MRP3 protein (*e.g.*, PepI, PepII, PepIII or PepIV; ~1004.2 RU [residual units] of peptide was immobilized) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) balanced salt solution (HBSS) buffer, composed of 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.05% BIAcore surfactant P20. The scFv antibodies were diluted in HBSS buffer and passed over the streptavidin chip at concentrations of 25, 200 and 1000 nM. The flow rate was kept at 30 μl/min, and the injection time was 3 min. Binding constants were calculated from the nonlinear fitting curve by using the BIAevaluation software (BIAcore).

ELISA with purified scFv antibodies

Binding specificity and crossreactivity of the purified scFvs were determined by ELISA assays for MRP3-derived peptides PepI, PepII, PepIII and PepIV. A 96-well plate was coated with 10 μg/ml of a specific MRP3-derived peptide conjugated with BSA as antigen *versus* BSA and other appropriate control antigens. The rest of the protocol was the same as described earlier except that the purified scFv antibodies were added in serial dilution to the wells, and the secondary antibody used was HRP-conjugated anti-His antibody (1:5000, Invitrogen).

Flow cytometry

For flow cytometric analysis of M25, M58 and M89 scFv antibodies to test the cell surface MRP3 molecule binding, either live neurospheres (D2159MG) or dissociated cells from xenografts (D2159MG, 6B-47MG, D633EP and D612EP)

were harvested (2×10^5/test), washed and blocked with 10% FCS. Adherent D54MG, D247MG and ovarian carcinoma cell lines (2×10^5/test) were harvested with 0.02% EDTA/PBS and fixed by resuspending them in 2–4 ml of 10% formaldehyde. After washing, the cells were blocked with 10% normal goat serum (NGS), with and without saponin, for 20 min. Saponin was added to facilitate the permeabilization of scFvs for examining the total surface receptors. Cells from all the cell lines and xenografts were incubated with corresponding scFv for an hour, on ice for live cells and at room temperature for fixed cells, and were washed twice and incubated with anti-histidine tag (C-terminus)-FITC-conjugated antibody (1:5000, Invitrogen) for an hour, on ice for live cells and at room temperature for fixed cells. Finally, live cells were suspended in PBS and were analyzed. Fixed cells were suspended in 0.5% paraformaldehyde-BSA and freshly analyzed by using a Becton Dickinson FACSort flow cytometer with CellQuest software (BD Biosciences, San Jose, CA).

Immunoreactive fraction determination

Immunoreactive fractions of the radioiodinated M25, M58 and M89 scFvs were determined according to the method described by Lindmo *et al.*[37] Purified recombinant scFvs (M25, M58 and M89) were labeled with ^{125}I by the Iodogen method, as described by Reist *et al.*,[38,39] to a specificity of 1–3 μCi/μg and purified by gel filtration on a 10-cm PD-10 Sephadex G-25 (Pharmacia, Pfizer, New York, NY) column.[40]

Fractions of immunoreactive scFvs were calculated by linear exploitation of infinite antigen excess. For these procedures, 1 ml of streptavidin-coated beads (MPG Streptavidin, Pure Biotech, LLC, Middlesex, NJ) was coated with 100 μg of biotinylated PepI or PepII and BSA to measure nonspecific binding. Beads were then rinsed to remove unbound peptides and resuspended in the original volume of Lindmo buffer (115 mM phosphate buffer of pH 7.5, 0.05% BSA and 0.05% Brij detergent [Sigma]). Triplicate aliquots of beads in increasing volumes (25, 50 and 100 μl) were incubated for 45 min at room temperature with 50 μl of Lindmo buffer containing 5 ng of each of the labeled scFv antibodies. Final volume was adjusted with Lindmo buffer. Beads were washed and separated by using a magnetic separator, and pellets and supernatants were counted in a gamma counter. Specific binding was calculated by subtracting nonspecific binding to BSA-coated beads from the binding of M25, M58 or M89 scFv separately. Plotting the total activity divided by the specifically bound activity *versus* the reciprocal of the antigen concentration yielded a linear plot, the intercept of which represents the inverse of the immunoreactive fraction.

Internalization assay

Internalization assays were performed with D2159MG, D54MG and D247MG cell lines, plated at a density of 5×10^5 cells per well in NSCM or ZO medium and incubated at 4°C for 30 min. The medium was removed from each well and replaced with fresh cold medium containing 1 μg/ml of radiolabeled scFv antibody. After 1 hr of incubation at 4°C, the medium was removed, and wells were washed twice with 1 ml of cold medium to remove unbound activity. Fresh medium was added to the wells, and plates were placed in an incubator at 37°C for different time periods (0, 1, 2, 4, 8, 20 and 24 hr). After each time point, supernatant was removed, and cells were washed twice with medium (pH 2.0) for 10 min to remove surface-bound activity. Finally, the cells were solubilized in 0.5 ml of 0.5 N NaOH (internalized fraction). Each supernatant sample was precipitated by 12% trichloroacetic acid to calculate acid-precipitable counts. All the fractions of the supernatant, acid washes and the cell pellets were counted in a gamma counter and were expressed as the percentage of activity initially bound to the cells after 1 hr of incubation at 4°C.

Immunohistochemistry

Tissue microarrays (TMAs) made of tissues from GBM patients were used for immunohistochemical studies. TMAs were constructed by obtaining cylindrical tissue specimens from regular paraffin blocks and then assembling them into a single block. Immunostaining for MRP3 was performed as previously described.[41] Briefly, TMAs were deparaffinized in xylene, rehydrated in a graded alcohol series and washed with PBS. Antigen retrieval was performed by placing the slides in 50 mM citrate buffer (pH 6.0) and microwaving it, first for 2 min at full power or until the buffer started to boil and then continuing to microwave for 8 min at 30% power, followed by cooling for 15 min and 2–3 PBS washes of 5 min each. Quenching of endogenous peroxidase was done by incubating the sections in 1% H_2O_2 in methanol for 10 min. Sections were washed in PBS and blocked for 1 hr at room temperature in 20% NGS. Sections were then incubated with purified M25 and M58 scFvs separately in 1% BSA-PBS overnight at 4°C. Primary antibody was washed away with PBS, and TMAs were incubated with Penta-His secondary antibody (Qiagen, Valencia, CA) in PBS (1:25) for 1 hr at room temperature. Tertiary antibodies were applied by using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Detection of bound tertiary antibodies was performed with diaminobenzidine for 1–5 min. Sections were then counterstained with hematoxylin and mounted. The TMAs were first scanned at 600× magnification using an Aperio Scanscope XT slidescanner. Automated image analysis was performed using Aperio Image scope (v9.1.19.1567) software and quantified with the Positive Pixel Count algorithm (v2).

Results

Isolation of MRP3-specific single-chain Fv antibodies

A naïve human scFv phage library, Mehta I, with 15×10^9 complexity,[34] was screened for single-chain antibody fragments specific for MRP3 protein. The homology between MRP3 protein and the other members of the human MRP protein family ranges from 33% with MRP5 to 58% with

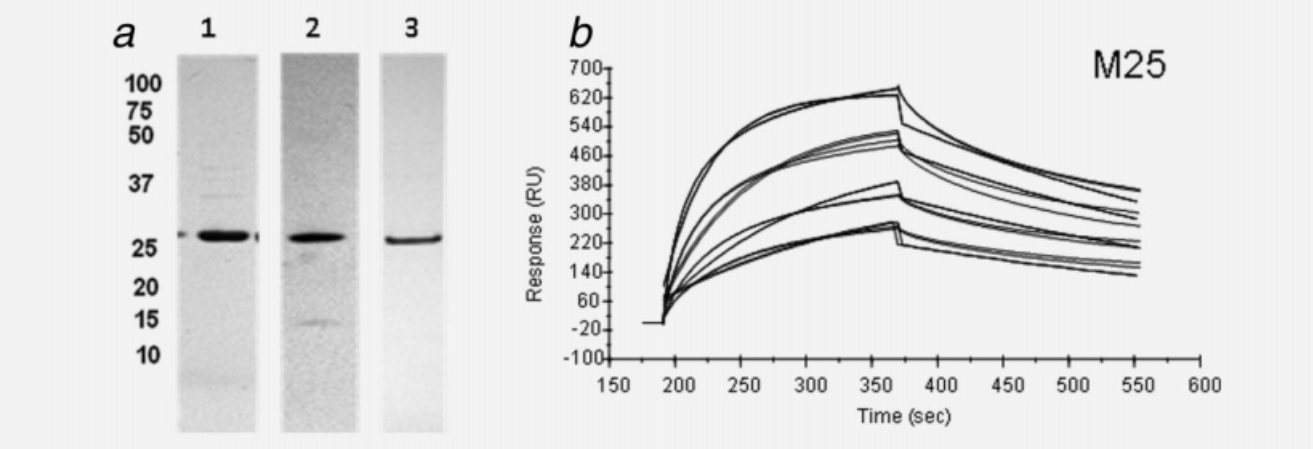


Figure 2. SDS-PAGE and BIAcore analysis of purified M25, M58 and M89 scFv antibody fragments after metal affinity chromatography. (*a*) Purified scFv proteins were analyzed by 4–12% SDS-PAGE. Lanes 1, 2 and 3 correspond to M25 (1 μg), M58 (1 μg) and M89 (500 ng), respectively. Molecular weight standards were in kDa. (*b*) Binding of M25, M58 and M89 scFvs was evaluated with a BIAcore instrument by immobilizing MRP3-derived, biotinylated PepI (for M25 and M58) and PepII (for M89) peptides. The sensogram in panel *b* shows the binding pattern of M25 scFv. The sensograms for M58 and M89 (not shown) showed the same pattern. The scFv antibodies were diluted in HBSS buffer and passed over the streptavidin-avidin chip at concentrations of 25, 200 and 1000 nM. The association and dissociation rates from the sensogram were k_{assoc} of 5.4×10^4 mol^{-1} $l^{-1}s$ s^{-1} and a k_{diss} of 1.9×10^{-3} s^{-1} for M25 scFv, k_{assoc} of 7.1×10^4 mol^{-1} l s^{-1} and a k_{diss} of 3.4×10^{-3} s^{-1} for M58 scFv and k_{assoc} of 5.1×10^4 mol^{-1} l s^{-1} and a k_{diss} of 5×10^{-3} s^{-1} for M89. K_A at binding equilibrium, calculated as $K_A = k_{assoc}/k_{diss}$, was 2.8×10^7 mol^{-1} l for M25 scFv, 2.1×10^7 mol^{-1} l for M58 scFv and 1×10^7 mol^{-1} l for M89 scFv.

MRP1. Hydrophobicity analysis of the MRP3 amino acid sequence indicates an organization of putative transmembrane domains resembling that suggested for MRP1. The precise membrane topology of MRP3 is complex and remains unclear. MRP3 is most closely related to MRP1, with 58% amino acid identity, and may have similar predicted topology of transmembrane segments. These proteins probably span the membrane 17–18 times, with the NH_2 terminus being extracellular.[42] After analyzing the topology and the antigenicity plot of the MRP3 amino acid sequence, we selected 4 peptides from extracellular domains that may elicit an immune response. As shown in Figure 1, 2 of the selected peptides, PepI and PepII, are from amino acids 1–20 and 21–32 of the amino terminus of the protein. The other 2 peptides, PepIII and PepIV, encompass amino acids 999–1013 and 1053–1069, respectively, and map to extracellular loop 6, 1 of the extracellular loops of the MSD3 domain of human MRP3 (Fig. 1).

To isolate scFv against the 4 selected extracellular epitopes, we screened the Mehta I human naïve scFv phage display library, which has a complexity of 15 billion independent clones. It was subjected to 3 rounds of panning with decreasing concentrations of biotinylated peptides, followed by elution of bound phages and reamplification of phages in *E. coli* strain TG1. A progressive and marked enrichment for phages was observed after each round of panning, with a total of 68- and 390-fold enrichment between the first round and third round of selection in the case of MRP3-derived PepI and PepII, respectively (Table 1). There was a total of 126- and 72-fold enrichment with PepIII and PepIV, respectively, in the third round of panning (data not shown).

A marked enrichment of specific phage clones was observed after the third round of panning, as exhibited also by polyclonal phage ELISA. Of 192 clones in each peptide screening for monoclonal phage ELISA, 54, 28, 7 and 38 phage clones exhibited binding to immobilized MRP3-derived PepI, PepII, PepIII and PepIV, respectively. DNA sequencing was performed with phage DNA of the specific clones, and the amino acid sequence was deduced. Putative clones were chosen for further analysis on the basis of their DNA sequence and their specificity as exhibited by monoclonal phage ELISA. Most of the clones against each peptide had an identical nucleotide sequence, as they converged after 3 rounds of enrichment. Among all positive clones, according to ELISA titer, M25 and M58 scFvs were selected as best for the PepI region of MRP3 protein, and M89, M23 and M17 were selected as best for the PepII, PepIII and PepIV regions of the MRP3 protein, respectively. On the basis of the binding analysis of scFv with cells expressing MRP3, we decided not to further pursue the evaluation of M23 and M17.

Characterization of anti-MRP3 scFvs

Expression and purification of M25, M58 and M89 scFv proteins. For expressing large quantities of pure recombinant scFv antibody in bacterial cells, scFv coding sequences were isolated from the pFarber phage display vector by NcoI and

NotI digestion and cloned into a pET 22b$^{(+)}$ vector in the same orientation. The scFvs expressed in bacterial cells were C-terminally tagged with histidine tag. All scFvs were produced in *E. coli* BL21 (DE3) gold by secretion and were purified from whole cell extracts by metal affinity chromatography using a C-terminal hexahistidine tag. Purified scFvs were analyzed on 4–12% SDS-PAGE (Fig. 2*a*), which revealed a major single band of ~27 kDa.

Binding kinetics, affinity measurements and specificity. Surface plasmon resonance technology implemented on a BIAcore instrument (BIAcore, Pharmacia) was used to characterize the binding kinetics and affinity of purified scFv antibody with immobilized biotinylated specific peptide. The association and dissociation rates from the sensogram were k_{assoc} of 5.4×10^4 mol^{-1} l s^{-1} and a k_{diss} of 1.9×10^{-3} s^{-1} for M25 scFv, k_{assoc} of 7.1×10^4 mol^{-1} l s^{-1} and a k_{diss} of 3.4×10^{-3} s^{-1} for M58 scFv and k_{assoc} of 5.1×10^4 mol^{-1} l s^{-1} and a k_{diss} of 5×10^{-3} s^{-1} for M89. K_A at binding equilibrium, calculated as $K_A = k_{assoc}/k_{diss}$, was 2.8×10^7 mol^{-1} l for M25 scFv, 2.1×10^7 mol^{-1} l for M58 scFv and 1×10^7 mol^{-1} l for M89 scFv (Fig. 2*b*).

To determine whether the recombinant scFvs could bind specifically to their corresponding MRP3-derived peptides, we examined soluble, purified scFv antibodies by ELISA assays. All scFvs reacted in a dose-dependent manner with their corresponding MRP3-derived peptides conjugated to BSA. They reacted only with the specific MRP3 peptide that was used to screen the library and did not crossreact with other MRP3 peptides (Fig. 3) or BSA.

Binding of M25, M58 and M89 scFv antibodies to MRP3-expressing tumor cells. To confirm that the purified, soluble M25, M58 and M89 scFvs bound to native MRP3 molecules expressed on the surface of tumor cells, we conducted indirect FACS of M25, M58 and M89 with a number of cell lines, such as D2159MG, 6B-47MG, D633EP and D612EP. Live tumor cells segregated from human glioma or ependymoma xenografts (D2159MG, 6B-47MG, D633EP and D612EP) were grown in ZO medium (Fig. 4*a*), and live neurospheres (D2159MG) were grown in NSCM (Fig. 4*b*). D2159MG neurospheres growing in NSCM showed much better binding than D2159MG cells growing in ZO medium or D2159MG cells derived directly from xenografts (Fig. 4*b*). Indirect FACS with fixed D247MG cells was also done under both permeabilized and nonpermeabilized conditions to look for total and cell surface binding (Fig. 4*c*). All of the cells from the xenografts and neurospheres exhibited a right shift in the peak, indicating binding of M25 and M58 to the surface of live cells, as shown in Figure 4; however, M89 did not exhibit binding to viable cells (data not shown). The shift associated with the binding of M25 was greater than the shift for M58 in all cell lines or neurospheres used and showed that the binding to cell surface is consistent with the affinity measured by the BIAcore biosensor (Figs. 4 and 2*b*). D247MG showed a shift in the peak both in permeabilized

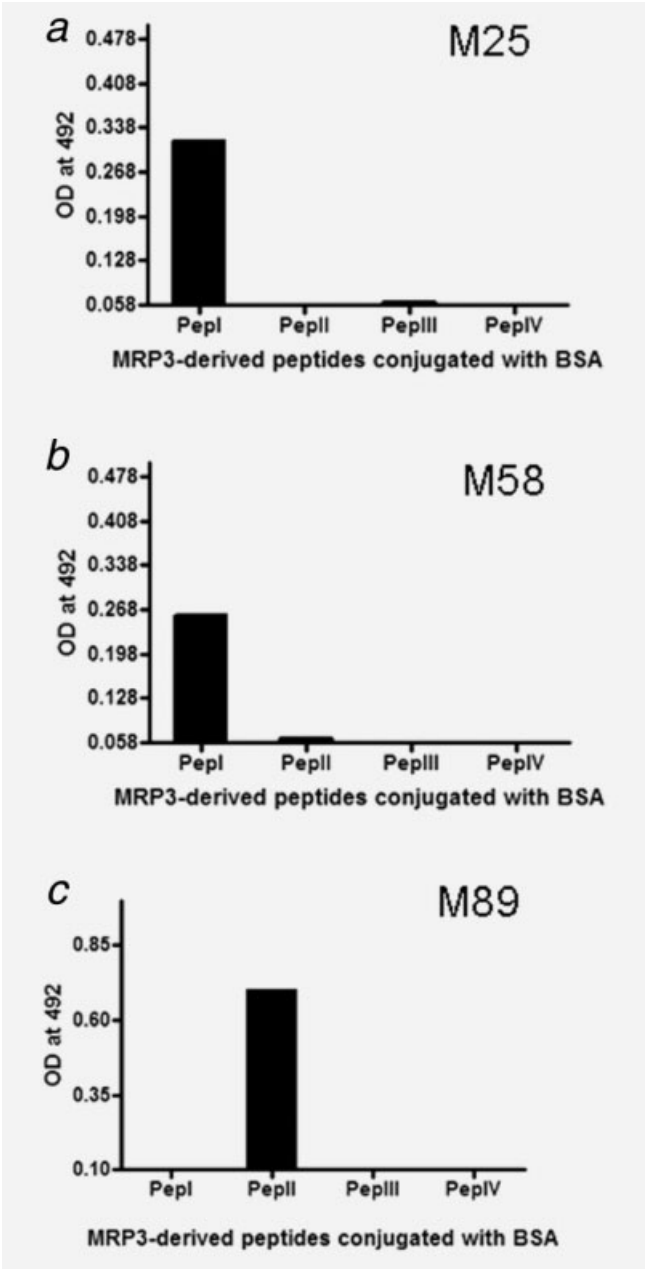


Figure 3. Binding specificity of anti-MRP3 scFvs. (*a*) M25, (*b*) M58 and (*c*) M89 scFvs were analyzed by ELISA with soluble, purified recombinant scFvs. A 96-well ELISA plate was coated with 10 μg/ml of specific MRP3-derived peptides (PepI, PepII, PepIII and PepIV) conjugated with BSA as antigen. The purified scFv antibodies were added in serial dilution to the wells, and the secondary antibody used was HRP-conjugated anti-His-tag antibody. The M25 (*a*) and M58 (*b*) scFvs reacted only with PepI antigen and not with other MRP3-derived peptides (PepII, PepIII or PepIV), whereas the M89 scFv (*c*) reacted only with PepII antigen and not with other MRP3-derived peptides (PepI, PepIII or PepIV).

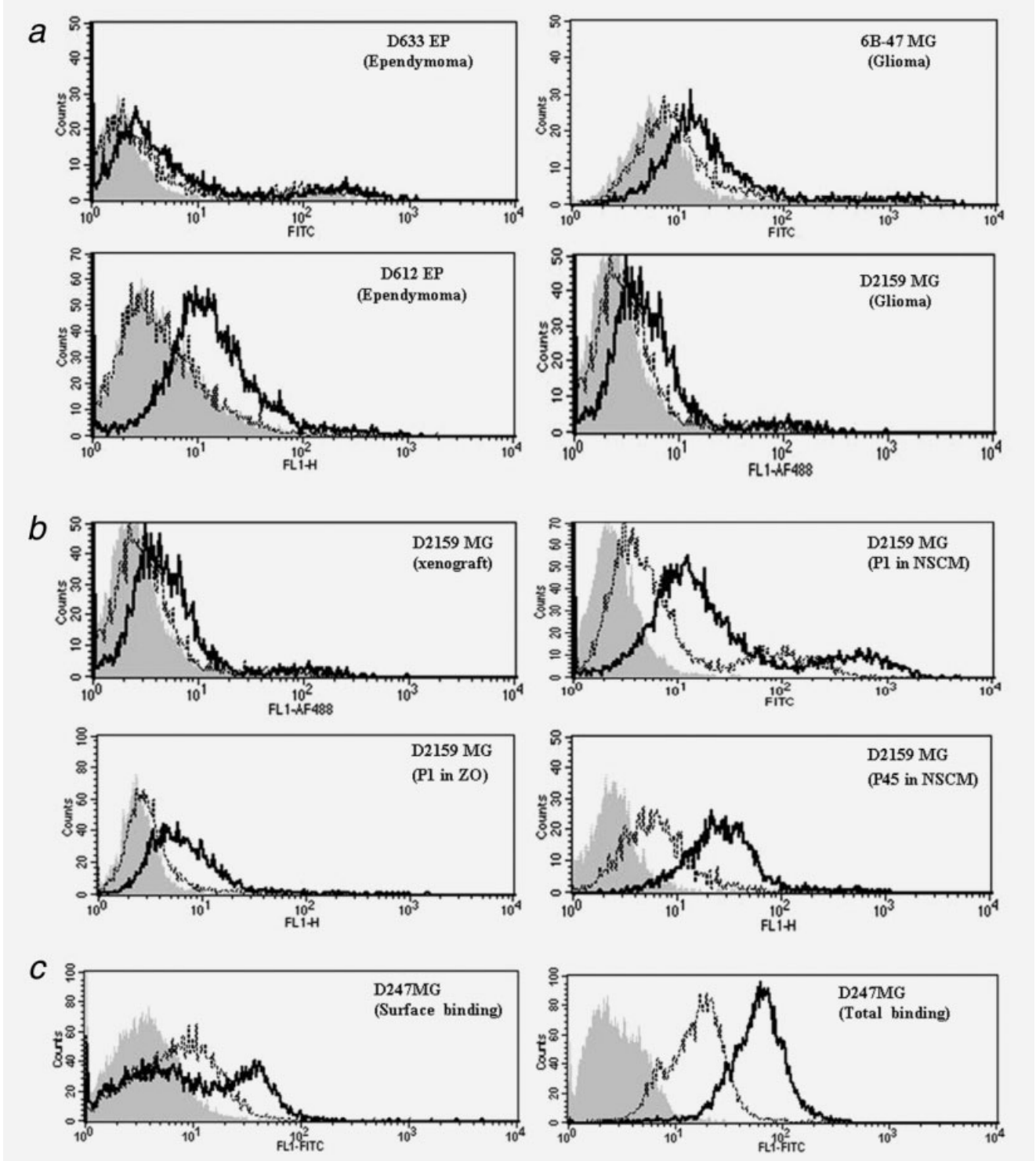


Figure 4. Detection of MRP3 on the surface of malignant glioma (MG) and ependymoma (EP) cells. (*a*) Indirect flow cytometric analysis of MRP3 expression in freshly isolated cells from D633 EP, D612 EP, 6B-47 MG and D2159 MG xenografts using purified M25 (solid black line) and M58 (dotted black line) scFv antibodies. Filled gray peaks are irrelevant scFv control. (*b*) Live D2159 MG neurospheres growing in NSCM at passage 1 and 45 exhibited better binding with M25 (solid black line) and M58 (dotted black line) than adherent D2159 MG cells growing in ZO medium (ZO; lower left panel) or D2159 MG cells from xenografts (upper left panel). Filled gray peaks are irrelevant scFv control. (*c*) MRP3 expression in permeabilized (total binding) and nonpermeabilized (surface binding) fixed D247 MG cells was done by using purified M89 (solid black line) and M58 (dotted black line) scFv antibodies. Filled gray peaks are irrelevant scFv control.

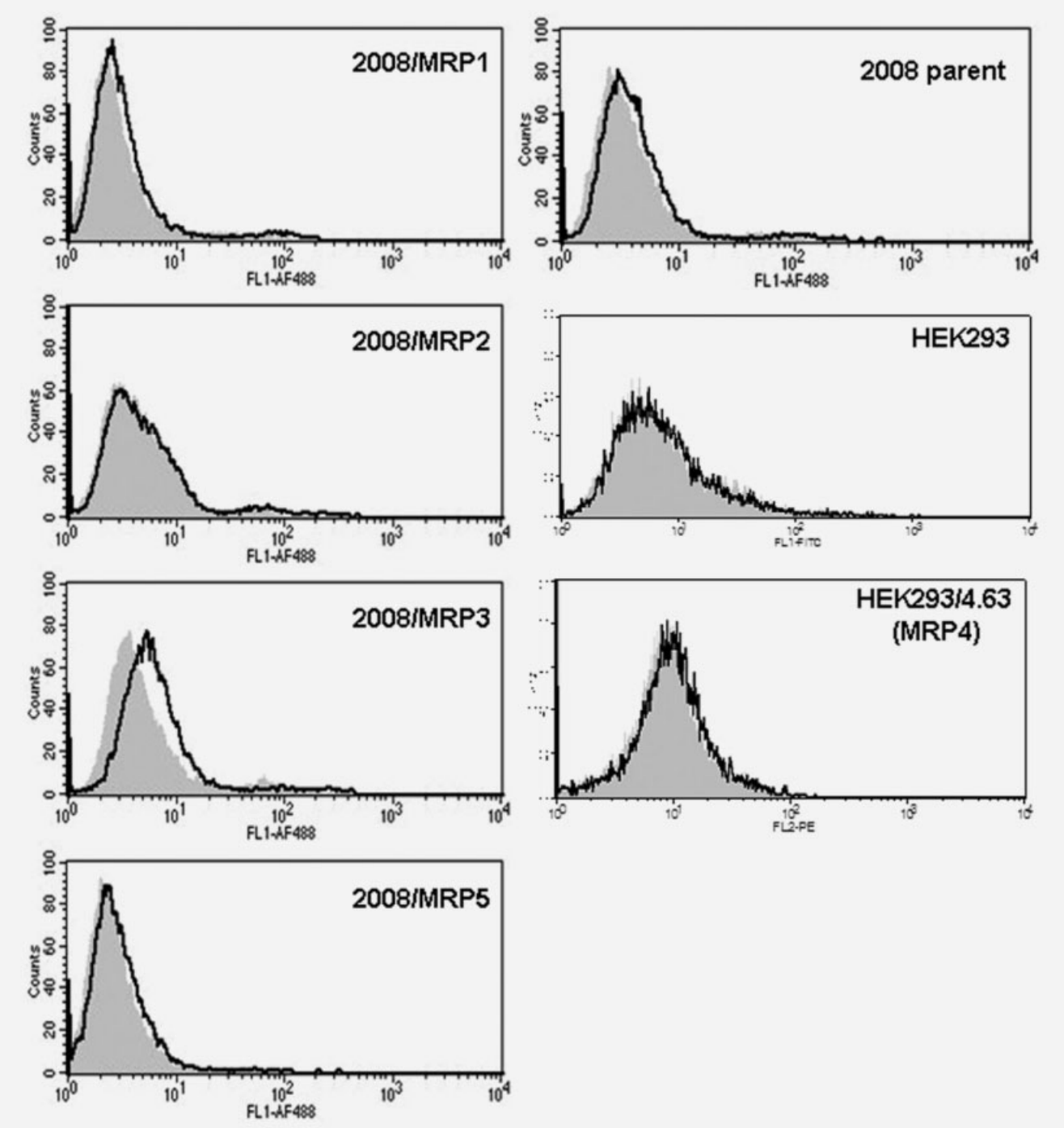


Figure 5. FACS analysis of MRP1–5 overexpressing cells with a purified M25 scFv antibody. Ovarian carcinoma cell line 2008 and its MRP transfectants, 2008/MRP1, 2008/MRP2, 2008/MRP3 and 2008/MRP5, as well as human embryonic kidney cell line HEK293 and its MRP4 transfectant HEK293/4.63, were used to confirm the specific binding of the M25 scFv antibody. Exponentially growing live cells were incubated with purified M25 scFv at 10 μg/ml followed by incubation with HRP-labeled anti-His tag secondary antibody. Filled gray peaks are irrelevant scFv control.

and in nonpermeabilized cells, indicating the binding of M58 and M89 scFv antibodies to surface MRP3 molecules under the fixed cell condition. Irrelevant control scFv was unreactive with all of the cell lines (Fig. 4*c*).

FACS analysis of anti-MRP3 scFv antibodies to viable stable transfectants expressing MRP1–5

To rule out the possibility of crossreaction with other members of MRP families, we analyzed the purified M25 scFv by

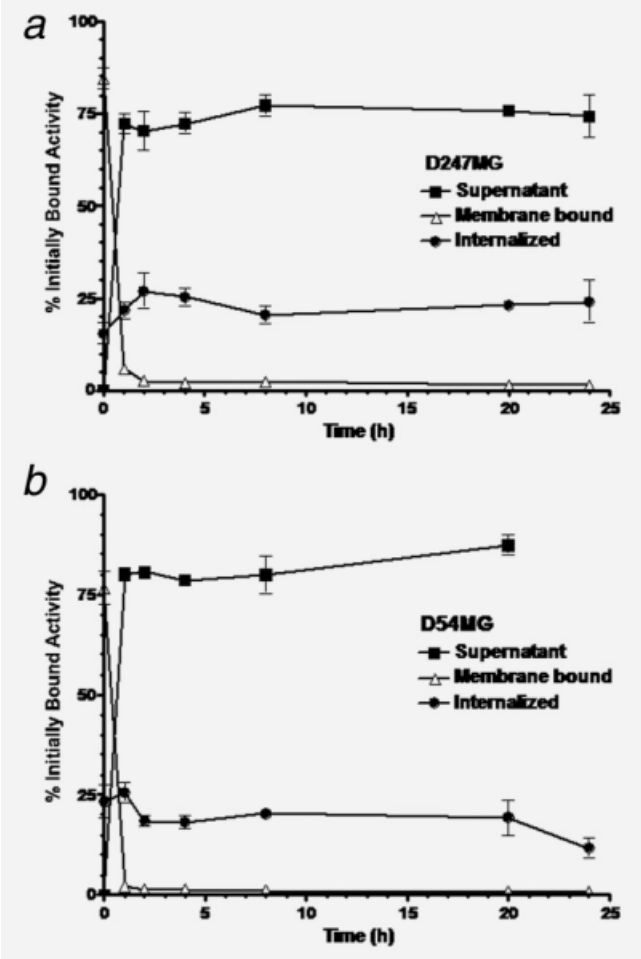


Figure 6. *In vitro* internalization of ^{125}I-labeled M89 scFv on (*a*) D247MG and (*b*) D54MG malignant glioma cells. The percentage of radioactivity initially bound to cells (membrane bound [Δ] or internalized [●]) and in the cell culture supernatant [■] is shown. A marked increase in radioactive counts was observed in the cell culture supernatant, with a slight decrease in counts associated with cell surface. The profile showed that the internalization of M89 scFv into D247MG cells gradually increased to a maximum percentage (26%) of intracellular counts at the 2-hr time point compared with 25% in D54MG cells at the 1-hr time point; 20% of the M89 scFv counts were detected intracellularly throughout the time course up to the 20-hr time point for both D54MG and D247MG. Points, average of triplicates; bar, SD.

FACS using ovarian carcinoma cell line 2008 and its transfectants expressing MRP1, MRP2, MRP3 or MRP5. We also analyzed the same purified M25 scFv by FACS using human embryonic kidney cell line HEK293 and its transfectants expressing MRP4 (HEK293/4.63). The analysis revealed that the binding of the M25 scFv antibody was very specific to the MRP3-expressing cells, with no crossreactivity to the cells expressing other MRPs, as shown in Figure 5. M58 and M89 also showed no crossreactivity with other members of MRP-family proteins expressed on the 2008 cell line (data not shown).

Radioiodination and immunoreactivity of purified scFv antibody

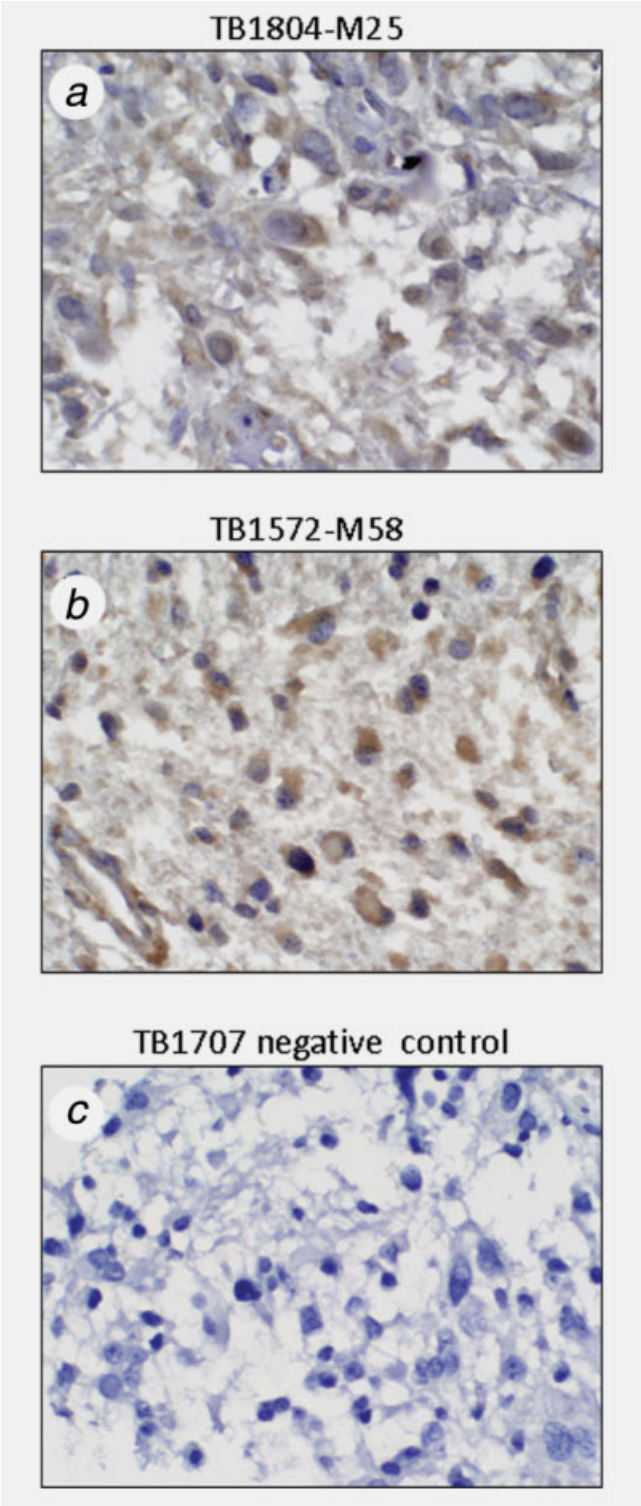
Purified M25, M58 and M89 scFv antibodies were radiolabeled with ^{125}I by the Iodogen method and purified by gel filtration. Radiolabeled scFvs were analyzed by gel filtration HPLC using a TSK2000SW column (Tosoh Bioscience, Montgomeryville, PA) and by surface plasmon resonance (BIAcore). Radiolabeling yields were in the range of 56–76% for the M25, M58 and M89 scFv antibodies, and the BIAcore analysis showed almost identical kinetics and affinities as were measured for the corresponding nonradiolabeled scFvs. The immunoreactivity of ^{125}I-labeled M25, M58 and M89 scFvs was measured by using magnetic beads coated with biotinylated PepI (for M25 and M58) or PepII (for M89). Immunoreactive fractions of the labeled M25 and M58 scFv antibodies were 32 and 52%, respectively, and for M89 the corresponding fraction was 69% (data not shown).

Internalization and processing of anti-MRP3 scFvs

Internalization of anti-MRP3 M89 scFv fragments was conducted according to the method described by Reist *et al.* (1995)[38] using D247MG and D54MG tumor cells to determine whether scFv bound to MRP3 internalizes at physiological temperature. D247MG and D54MG cells were incubated with purified M89 scFv for 1 hr at 4°C to allow scFv to bind to the cell surface. The cultures were then adjusted to 37°C, and cell-associated radioactivity was measured at 0, 1, 2, 4, 8, 20 and 24 hr. Percentages of radioactive counts for cell culture supernatant, cell surface and intracellular compartments for different time points were plotted for both cell lines, D247MG and D54MG (Figs. 6*a* and 6*b*, respectively). Cell-surface-associated counts for M89 scFv showed a rapid drop, accompanied by an increase in counts in the cell culture supernatant (Fig. 6). During early time points, from 1 to 4 hr, ~70–80% of the associated counts were released to the cell culture supernatant. The radioactivity of scFv associated with the cell culture supernatant increased and accounted for 80% of the radioactive counts for D247MG cells and 90% for D54MG cells of the total cpm recovered after 24 hr at 37°C (Fig. 6). The profile showed that the internalization of M89 scFv into D247MG cells gradually increased to a 26% maximum percentage of intracellular counts at the 2-hr time point when compared with 25% for M89 scFv in D54MG cells at the 1-hr time point. For both D54MG and D247MG, 20% of the M89 scFv counts were detected intracellularly throughout the time course up to the 20-hr time point (Fig. 6). In addition, M25 exhibited 20% internalization into D2159MG neurospheres, and M58 showed 33% internalization into D54MG cells (data not shown).

Immunohistochemical analysis

To confirm the specificity of anti-MRP3 scFvs M25 and M58, we examined the expression of MRP3 in paraffin-embedded sections of GBM patient tissue immunohistochemically using purified M25 and M58 scFv antibodies. Immunohistochemistry of M25 revealed a membranous/cytoplasmic binding pattern in 23 (45%) of 51 malignant gliomas. The labeling index varied from 5 to 80% of tumor cells among the positive biopsy cases analyzed (Fig. 7). Immunohistochemistry of M58 also revealed a cytoplasmic and membranous staining pattern in 37 (79%) of 47 malignant gliomas tested. The labeling index among positive cases with M58 scFv-stained sections varied from 5 to 40%. M58 revealed more robust reactivity with GBM cells than was seen with M25.



Discussion

GBM is the most prevalent and most aggressive type of brain tumor. In spite of advances in surgery, radiotherapy and chemotherapy, most patients die within a year of diagnosis. GBM cells express different levels of certain cell surface proteins than the normal surrounding brain cells. Many antigens, such as glycoprotein transmembrane nmb (gpnmb), epidermal growth factor receptor (EGFRwt) and its glioma-associated deletion variant (EGFRvIII) and the interleukin 13 receptor α2 isoform (IL-13Rα2), have been identified whose expression is clearly upregulated in brain tumor cells. MRP3 has been identified as one of the GBM-associated molecules,[21] being expressed at high levels in GBM but not in normal brain cells.[31] Therefore, MRP3 is a potentially useful tumor-associated antigen and prognostic predictor for immunotherapeutic approaches for malignant gliomas.

Using recent advances in gene technology, we have developed single-fragment chain human antibodies to target GBM tumors.[31,43] We isolated scFv antibodies specific for extracellular MRP3 epitopes by screening a human naïve phage display library with 15×10^9 complexity.[35] These scFv antibodies meet the prime criterion for being a successful therapeutic or diagnostic agent for GBM therapy—high affinity for the target antigen, specificity toward antigen, binding to the extracellular epitope and efficient internalization into cells.

As localization of the target antigen is an important factor in the selection of a potential target for GBM

Figure 7. Immunohistochemical demonstration of M25 and M58. Tissue microarrays (TMAs), made of paraffin-embedded sections of tissues from GBM patients, were used for immunohistochemical analysis using purified M25 and M58 scFvs. TMAs were deparaffinized, and antigen was retrieved by boiling the citrate buffer. After washing and blocking with NGS, sections were incubated with purified M25 and M58 scFvs separately in 1% BSA/PBS overnight at 4°C. Anti-penta-His tag was used as secondary antibody, followed by incubation with tertiary antibody from Vectastain Elite ABC kit (Vector Laboratories). Sections were counterstained with hematoxylin. (*a*) Purified M25 scFv showed binding to malignant astrocytic cells in a membranous cytoplasmic pattern, by binding to ~50% of the tumor cells (M25, ×40). (*b*) IHC of M58 also revealed a cytoplasmic/membranous staining pattern in malignant glioma biopsies. In IHC testing, more tumors (79%) demonstrated more frequent cellular staining with M58 than with M25. (*c*) IHC with an irrelevant scFv antibody at matching concentrations was entirely negative. Magnification, ×600.

immunotherapy, we selected 4 peptides from the extracellular NH_2 terminus and 1 from the extracellular loop of MRP3 protein, on the basis of an antigenicity plot and a hydrophobicity analysis. Four biotinylated peptides (PepI, PepII, PepIII and PepIV) were used to screen the phage display library, and we assessed the specificity and sensitivity of all purified scFv antibodies by ELISA assays. There was no crossreactivity observed among the scFvs isolated from different peptides derived from MRP3 protein. For example, the M25 scFv reacted only with immobilized PepI antigen, and no crossreactivity was observed with PepII, PepIII or PepIV. Positive phage clones were sequenced after selection against each of the peptides derived from MRP3. After 3 rounds of enrichment, we observed that most of the clones against each peptide (PepI, PepII, PepIII or PepIV) had merged to an identical nucleotide sequence. The M25 and M58 scFvs were selected as optimal binders for the PepI region of MRP3 protein and M89 for the PepII region.

BIAcore analysis of purified recombinant scFvs on a microsensor chip showed binding affinity in the range of 1×10^7. This level of binding affinity for first-generation, single-chain variable fragments is sufficient for tumor targeting when the antigen is expressed in notably high levels.[44] But for moderately or less expressed antigens, antibody fragments with higher affinities are preferred.[45]

We used FACS to further investigate the binding of purified M25, M58 and M89 scFvs to native MRP3 molecules expressed on the surface of GBM and ependymoma tumor cells, neurospheres and cells segregated from xenografts. All 3 scFvs showed a different binding pattern with live cells. Of the 3 scFvs selected, M25 was able to exhibit greater specific binding with all the cell lines and xenografts used by FACS and was able to show binding specifically with ovarian carcinoma cell line 2008 transfectants expressing MRP3, but there was no binding activity with transfectants expressing MRP1, MRP2, MRP4 or MRP5. M25 and M58 showed a greater shift with D2159MG neurospheres growing in neural stem cell medium than with D2159MG growing in ZO with FCS, which suggests that MRP3 may be expressed more in cells growing in neural stem cell medium than in those growing in differentiation medium (ZO with FCS). M58 and M89 did not show any crossreactivity with ovarian carcinoma 2008 transfectant cell lines, including MRP1, MRP2, MRP4 and MRP5. We also analyzed M58 and M89 under fixed cell conditions in permeabilized and nonpermeabilized cells and found that both scFvs bound specifically to only MRP3-expressing D247MG cells. Additionally, the data support the immunohistochemistry evaluation, where M25 (45%) and M58 (79%) showed staining with MRP3-expressing glioma biopsy tissues. However, we did not find any staining with M89 by immunohistochemistry. Normal brain samples did not show any binding with any of the scFvs tested.

Immunoreactive fractions for M25, M58 and M89 scFvs were measured. M89 scFv produced a significantly higher immunoreactive fraction than M25 and M58. We also determined the amount of scFv that was internalized into the cells. This measure is also an important criterion for choosing a scFv antibody as a therapeutic agent, as internalization is required for binding and cytotoxicity of immunotoxins.[46,47] An *in vitro* internalization assay for the purified M89 scFv demonstrated significantly higher amounts of ^{125}I-labeled M89 scFv internalized by D54MG and D247MG cells during the first 2 hr of the assay, and a good percentage of it remained internalized throughout the assay. D54MG exhibited up to 25% internalization of M89 scFv within the first hour, and then it dropped slowly to 18% between the 2- and the 20-hr time points. For D247MG cells, counts for the internalized M89 scFv dropped to about 16% after 20 hr. The pattern of internalization was very similar to that of a previously reported anti-EGFRvIII monoclonal antibody, L8A4, by HC2 20 d2 cells expressing EGFRvIII.[40,43]

In comparison with other anti-MRP3 antibodies, M25, M58 and M89 are completely human single-chain antibodies against cell surface epitopes. To our knowledge, these are the first scFv antibodies isolated against extracellular epitope of MRP3 from a human naïve phage display library. These scFvs, displaying good affinity and specificity, can be used as radiolabeled single-chain fragments for immunotargeting of malignant brain tumors and potentially other MRP3-positive tumors.

In conclusion, we have successfully isolated M25, M58 and M89 anti-MRP3 scFv antibodies that bind selectively to external MRP3 epitopes that are expressed on brain tumor cells. We found no crossreactivity with normal brain cells and found good binding affinity for first-generation scFv isolates. Because of their good affinity and specificity, these scFvs can be used as a vehicle for radioisotopes or other cytotoxic drugs to target MRP3-expressing cancer cells. Moreover, the affinity of selected antibodies can be improved by affinity maturation in clones developed through construction of mutant antibody libraries. Significantly, these scFvs, with good immunoreactivity and internalization into MRP3-expressing malignant cells, are excellent candidates for immunotoxin development. Thus, these MRP3-specific scFvs are potentially valuable reagents for brain tumor diagnosis and targeted therapy.

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