4 Materials and Methods

4.1 General materials

4.1.1 Nemod Hybridoma clones

A70-C/C8 IgMκ: is specific for Lewis Y (Cao, Merling et al. 2001).

A70-A/A9 IgG1κ: recognises Lewis Y and Lewis b (unpublished antibody).

A51-B/A6 IgAκ: recognising H disaccharide, H type 2, and Lewis Y (unpublished antibody).

A46-B/B10 IgMκ: recognising H type 2 and Lewis Y (Karsten, Pilgrim et al. 1988).

A78-G/A7 IgMκ: is specific for TF (Karsten, Butschak et al. 1995).

A63-D/B12 IgMκ: recognising H type 2 and Lewis Y (unpublished antibody).

A69-A/E8 IgMκ: recognising Globo H (unpublished antibody).

A78-G/A7 IgMκ: recognises the Thomsen-Friedenreich (TF) disaccharide (Karsten, Butschak et al. 1995).

PankoMab IgG1κ: recognises the glycosylated MUC1 epitope APDTRPAP.

4.1.2 Antigens

PAA conjugates: Oligosaccharides conjugated to poly[N-(2-hydroxyethyl)acrylamide] (Lectinity Holdings, Inc, www.lectinity.com)

Asialoglycophorin from human blood type MN (Sigma-Aldrich, Steinheim, Germany).

Muc1-Tn glycopeptide: (APPAHGVTSAPDT(αGalNAc)RPAPGSTAPPAHGVTSA) (Biosyntan, Berlin, Germany)

Bovine histone H1 (Alexis Biochemicals, Heidelberg, Germany)

Human histone H1.2 (Alexis Biochemicals, Heidelberg, Germany)

Natural mixture of bovine histones (Alexis Biochemicals, Heidelberg, Germany)

BSA: Albumin Fraktion V, Bovine (Carl Roth GmbH & Co., Karlsruhe, Germany)

4.1.3 Phage

Griffin.1, TomlinsonI and TomlinsonJ scFv libraries from the MRC, Cambridge Centre for Protein Engineering, Cambridge England were kindly provided by Prof. Greg Winter and Dr. Ian Tomlinson, respectively. KM13 Helper phage (Kristensen & Winter, 1998) was supplied by Dr. Peter Kristensen and produced as described in the MRC phage display protocols.

4.1.4 Bacteria

E. coli TG1: Suppressor strain was used for cloning and for amplification of phage between rounds of panning and expression of protein (Supplied by MRC, Cambridge, UK)

4.1.5 Cell lines

SW 480 (ATCC No CCL-228, colorectal adenocarcinoma), HEK293 (ATCC No CRL-1573, Kidney cells transformed with adenovirus 5 DNA), MCF-7 (ATCC No HTB-22, breast adenocarcinoma) and LS174T (ATCC No CL-188, colorectal adenocarcinoma), T47D (ECACC No 85102201, breast ductal carcinoma), ZR-75-1 (ATCC No CRL-1500, breast ductal carcinoma), OVCAR-3 (ATCC No HTB-161, ovary, epithelial, adenocarcinoma), HepG2 (ATCC No HB-8065, liver, hepatocellular carcinoma), HCT 116 (ATCC No CCL-247, colon, colorectal carcinoma), HCT-15 (ATCC No CCL-225, colon, colorectal carcinoma), MDA-MB-435S (ATCC No HTB-129, mammary gland; breast; duct; from metastatic site: pleural effusion ductal carcinoma), JEG-3 (ATCC No HTB-36, placenta, choriocarcinoma), MT-3 (DSMZ No ACC 403, human breast carcinoma), SK-OV-3 (ATCC No HTB-77, ovary, metastatic site, ascites adenocarcinoma), HT29 (ATCC No HTB-38, colon, colorectal adenocarcinoma), U266 (ATCC No TIB-196, B lymphocyte; plasmacytoma, myeloma), H184A1 (non-tumorigenic breast epithelial cell line) (kind gift from Dr. Dörken MDC), KG-1: Homo sapiens, bone marrow, acute myelogenous leukaemia, K562 (DSMZ ACC 10, human myelogenous leukemia cell line), NM-D4, NM-F9: Derived from K562 by selection for TF.
COS-7 cells (ATCC No CRL-1651) or CHO cells (ATCC No CCL-61) were transfected with the plasmids encoding a κ and γ or μ chain of interest by lipofectamine^{TM} 2000 (Invitrogen GmbH, Karlsruhe, Germany) transfection according manufactures instruction.

4.1.6 Vectors

pHEN2 contains a Lac Z promotor, PelB leader, and sequences coding for hexahistidine and myc affinity tags.
pKBJ3 vector enable expression of phage p3 DI fusion proteins (Jensen, Larsen et al. 2002).
Human γ, μ, and κ expression vectors were derived from pcDNA3 and are IP of Nemod Immunotheraphie GmbH.

4.1.7 Primers

All primers were synthesised according to the degenerate primer sequences of Zhou and co-workers (Zhou et al., 1994), but adapting the restriction site sequences (NcoI, XhoI, ApaLI, and NotI respectively) in pHEN2. Primer mixtures were always 10μM. Additional primers used for construction of CC8 scFv with 1aa linkers are:
GGGCCCGAAGACAACGC**Tgcagagacagtgaccagagt**
GGGCCCGAAGACAAAGCG**gatattgtgctaactcagtct**

4.1.8 Antibodies and lectin

Anti-Human/HRP Goat anti-Human Ig HRP (Jackson Immunoresearch Lab. Inc, PA, USA).

Anti-Mouse/HRP: P 0260 Rabbit Anti-mouse HRP, Ig (DAKO, Glostrup, Denmark).

Anti-M13/HRP: (Amersham Biosciences, Freiburg, Germany).

Anti-p3/HRP: PSKAN3 (MoBiTec, Göttingen, Germany).

Anti-His/HRP: Tetra HisTM HRP conjugated (Qiagen GmbH, Hilden, Germany).

Anti-Myc: 9E10, Mouse Ascites Fluid (Sigma, Saint Louis, Missouri, USA).

Anti-Human κ: Mouse anti-Human κ chain (BD Bioscience, Heidelberg, Germany).

Anti-IgM: Goat anti-Mouse IgM (Jackson Imm. Lab. Inc, PA, USA).

Anti-Ig: Goat anti-Mouse Ig (Sigma-Aldrich, Steinheim, Germany)

Anti-Ig: anti-Mouse Ig AP (Sigma-Aldrich, Steinheim, Germany)

Anti-Mouse IgG/HRP: Goat anti-Mouse IgG (Fcγ) HRP (Jackson Imm. Lab. Inc, PA, USA).

Anti-Human IgG/HRP: Goat anti-Human IgG, $F(ab)_2$ HRP (Jackson Imm. Lab. Inc, PA, USA).

Anti-Human IgM/HRP: Affinipure anti-Human IgM Fcμ5 HRP (Jackson Imm. Lab. Inc, PA, USA).

Anti-Mouse IgG/POD: Goat anti-Mouse IgG (Fcγ) POD (Jackson Imm. Lab. Inc, PA, USA).

Anti-Human IgG/POD: Goat anti-Human IgG, $F(ab)_2$ POD (Jackson Imm. Lab. Inc, PA, USA).

Anti-Mouse IgG Cy3: Goat anti-Mouse IgG (Fcγ) Cy3 (Jackson Imm. Lab. Inc, PA, USA).

Anti-Mouse IgM Cy3: Goat anti-Mouse IgM (μ) Cy3 (Jackson Imm. Lab. Inc, PA, USA).

Anti-Mouse Ig Cy2: Goat anti-Mouse Ig Cy2 (Sigma-Aldrich, Steinheim, Germany).

Anti-Human IgG: Anti-Human IgG $(Fab)_2$ Cy3 (Jackson Imm. Lab. Inc, PA, USA).

Anti-Mouse IgG/M-Cy3: Goat Anti-mouse IgM + IgG Cy3 (Jackson Imm. Lab. Inc, PA, USA).

Streptavidin-Cy3 (Jackson Imm. Lab. Inc, PA, USA).

Anti-histone H1: (Dianova GmbH, Hamburg, Germany).

UEA I-Biotin: Ulex Europeaus A I Biotin (Sigma-Aldrich, Steinheim, Germany).

Mouse IgG1κ: Plasmocytoma-derived mouse antibody MOPC-21 IgG1κ (Sigma-Aldrich, Steinheim, Germany).

Mouse IgMκ: Plasmocytoma-derived mouse antibody TEPC 183 IgMκ (Sigma-Aldrich, Steinheim, Germany).

Mouse IgAκ: Plasmocytoma-derived mouse antibody TEPC 15 IgMκ (Sigma-Aldrich, Steinheim, Germany).

Anti-Mouse IgG1: polyclonal Rabbit IgG1 (Abcam, Cambridge,UK).

Anti-Mouse IgG2a: polyclonal Rabbit IgG2a (Abcam, Cambridge,UK).

Anti-Rabbit POD: Goat anti-Rabbit antibody (DAKO, Glostrup, Denmark).

Polyclonal affinipure goat anti-mouse IgM (Jackson Imm. Lab. Inc, PA, USA).

4.2 Phage display protocols

For extended protocols for selection of phage display libraries see:

http://ww.mrc-cpe.cam.ac.uk/g1p.php?menu=1808.

http://www.ghmp.mrc.ac.uk/geneservice/reagents/products/datasheets/scFv/tomlinsonIJ.pdf.

4.2.1 Phage amplification and precipitation

Production of phage from the bacterial library stock or from bacteria, which were infected by eluted phage from the selections (see below), was induced by superinfection with the helperphage KM13. Bacterial cultures in the log phase (OD_{600}= 0.5-0.6) were infected with helperphage at a ratio of 1:20 (number of bacterial cells: phage particles) for 30min at 37°C without shaking. Media was changed to 2xTY 100µg/ml ampicillin (Amp), 25µg/ml kanamycin (Kan) grown overnight at 30°C for phage production. Phage were purified from the supernatant by polyethylene glycol (PEG) precipitation and resuspended in PBS (phosphate-buffered saline: 25mM NaH_2PO_4, pH 7.0, 125mM NaCl).

4.2.2 Selections

Prior to selections several blocking agents were screened to assess their influence on the binding of the idiotypic antibody to the antigen. Selections were performed by immobilisation of (idiotype, Ab1) antibodies on either immunotubes or magnetic beads as follows:

Immunotubes: Three mls of each purified mAbs were incubated in immunotubes (MaxiSorp, Nunc, Wiesbaden, Germany) overnight at 4°C (40µg/ml in PBS for the first, and 10µg/ml for the second and third round of selection). The mAb-coated tubes were washed and blocked with 30% FCS/RPMI or 2% MPBS for 2h. For the first round 5 x 10^{12} t.u. of the phage library (Griffin.1 or Tom I+J), rescued by KM13, were used containing statistically 500 copies of each scFv per selection for TomI+J and 50 for Griffin.1 libraries. The libraries were selected for their anti-idiotypic binding to the mAb in a total volume of 3ml 10% FCS/ RPMI or 2% MPBS for 2h at RT. Prior to incubation with the mAb the phage were preincubated with their blocking agent. In the first round of selection the tubes were washed 8 times with 10% FCS/RPMI or 2% MPBS and 8 times with PBS, 0.1% (v/v) Tween20 (TPBS), and in the second round 16 times with both buffers. In each selection the tubes were finally washed twice with PBS before the phages were eluted as described below.

Magnetic beads: Purified mAbs (1.5µg/ 200µl beads) or supernatants of hybridoma cultures (6ml/ 200µl beads) were immobilized on mouse-IgG-specific (DB M-280, sheep anti-mouse IgG) or mouse-IgM-specific (DB M-450, rat anti-mouse IgM) magnetic beads (Dynal, Hamburg, Germany) for >2h at RT. After washing with TPBS the beads were blocked with either 30% FCS/RPMI or 4% MPBS for 1h at RT. All tubes were preblocked. Further steps were performed as with immunotubes. Prior to elution the beads were transferred to a new tube.

Elution and production of phage: The phage were eluted with 1-2ml (immunotubes) or 450µl (magnetic beads) of the indicated antigen (50 or 100µg/ml) in 50mM Tris pH 8, with 1mM $CaCl_2$, for 0.5 to 2h at RT and transferred to a new blocked tube. Trypsin (TPCK-treated, Sigma) in 50mM Tris pH 8, 1mM $CaCl_2$) was added to 1mg/ml in 0.5ml and incubated for 15min at RT. Alternatively, bound phage were eluted with: (1) The antigen alone in 50mM Tris pH 8, 1mM $CaCl_2$ for 0.5 to 2h; (2) 50mM glycine buffer pH 2.2 for 30min; (3) 50mM glycine buffer pH 2.2, followed by adjusting the pH with Tris buffer to pH 8, adding 1mM $CaCl_2$ and 1mg/ml trypsin, and incubating for 15min; or (4) only with trypsin directly from the beads using the same conditions as above.

Eluted phage were used to infect 9.5ml log phase *E.coli* TG1 for 30 min at 37°C. Dilution series were made for titration. The remaining cells were spun down, plated on 2 x TYE plates containing 0.1mg/ml Amp and 1% Glc,

and incubated overnight at 30°C. Colonies were picked singly for monoclonal cultures into 96 well plates (Nunc) with 100µl 2xTY containing 100µg/ml Amp and 1% Glc per well. For further selections, the colonies were scraped off the plate into 2xTY, 15-20% glycerol, of this stock 50-100µl were inoculated into 2xTY, 100µg/ml Amp, 1%Glc, and grown to log phase. The production of phage was done as described above.

Sequencing of scFv genes. Clones from the frozen glycerol stocks were amplified by PCR using Taq polymerase. Primers were LMB3 (CAG GAA ACA GCT ATG AC) and Fdseq1 (GAA TTT TCT GTA TGA GG). 50µl each were reacted in 25 cycles (94°C for 1min, 50°C for 1min, 72°C for 2min) after initial 10min at 94°C. Products were purified by the QIAquick PCR purification kit (QIAgen, Hamburg, Germany), and aliquots were analysed on 2% agarose gels. Primers for DNA sequencing were FOR_LinkSeq (GCC ACC TCC GCC TGA ACC) and pHEN-SEQ (CTA TGC GGC CCC ATT CA). Sequencing was done either by using fluorescent dideoxy chain terminators (Dye Terminator Sequencing Kit II, Amersham Pharmacia Biotech, Freiburg, Germany) or by automated sequencing.

4.2.3 Phage screening

Monoclonal phage particles were screened for binding in ELISA as described by Marks (Marks, Hoogenboom et al. 1991). ELISA plates were coated overnight at 4°C with 50µl antigen (10µg/ml in PBS), and blocked with 400µl 2% BSA-PBS for at least 60min at RT. Fifty microliter 2% BSA-PBS and 50µl phage supernatant were mixed in the well and incubated for 60min. Plates were washed six times with PBS and bound phage detected with anti-M13/HRP diluted 1:2500 in 1% MPBS. Following another six washes with PBS, the ELISA was developed according to the standard ELISA protocol.

4.3 Cloning

4.3.1 Sequencing

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

4.3.2 Cloning of DNA from hybridoma cells

mRNA was extracted from the hybridoma cells using the RNeasy midi kit (QIAGEN, Hilden, Germany) All buffers except the specified ones are from QIAGEN (Hilden, Germany) and are delivered with the RNeasy midi kit or Omniscript RT kit. Approx. $5x10^6$ cells were resolved in 600µl buffer RLT and the suspension is homogenised by passing it through a syring mounted with a 20-G (0.9mm) needle at least 5 times. 1 volumn of 70% ethanol was added to the homogenised lysate and the sample was mixed by pipetting. The lysate was applied on a RNeasy midi spin column and centrifuged. This step is repeated until all lysate has been passed over the RNeasy column. After washing with the buffers RW1 and RPE the column was eluted with 2x50µl RNase free water. The eluted sample was heated to 65°C for 2min. to disrupt secondary structure. 100µl Dynalbeads

Oligo $(dT)_{25}$ (Dynal, Hamburg, Germany) was washed twice in binding buffer (20mM Tris HCl, pH 7.5, 1M LiCl, 2mM EDTA) and resuspended in 100µl binding buffer. The heated sample and the resuspended Dynalbeads Oligo $(dT)_{25}$ were mixed thoroughly and the mix was incubated for 3-5min. with rotation for annealing. After annealing the Dynalbeads Oligo $(dT)_{25}$ was washed twice with buffer B (10mM Tris HCl, pH 7.5, 0.15 M LiCl, 1mM EDTA) and twice with RNase free water on ice with very gentle mixing to avoid tearing mRNA. cDNA was reversed transcribed by incubating the annealed Dynalbeads Oligo $(dT)_{25}$ in 40µl reverse transcriptase mastermix (4µl 10x buffer RT, 20 units RNase inhibitor, dNTP 0.5mM each –final concentration, 8 units Omniscript Reverse Transscriptase, RNase free water to 40µl) at 37°C for 60min. with rotation. After cDNA synthesis the Dynalbeads Oligo $(dT)_{25}$ are washed twice in TE buffer (20 mM Tris, 1 mM EDTA). The synthesized cDNA from the hybridomas served as template in two separate polymerase chain reactions (PCR), one for variable heavy chain using a mix of the VH for and back primers, and one for variable light chain using a mix of the VL for and back primers. The amplified genes were purified using the QIAGEN PCR purification kit, and digested overnight (NcoI and XhoI for VH and ApaLI and NotI for VL) and cloned into pHEN2. Subsequently the inserts were digested with various restriction enzymes needed for cloning into the Nemod human µ, γ, and κ expression vectors. The 1 amino acid linker scFv is cloned by making PCR on pHEN2 with the two appropriate VH and VL primers and two central primers introducing a BbsI restriction. The amplified genes were purified using the Qiagen PCR purification kit, and digested overnight with BbsI. The digests were separated on a 2% agarose gel, and the desired DNA fragments excised and extracted. The VH and VL were joined by ligation (T4 DNA ligase (NEB)), and amplified by a new PCR using the VH back and VL for primers. The new PCR fragment is digested with NcoI and NotI and ligated into NcoI-NotI cut pHEN2.

4.3.3 Construction of DI fusions

Phagemids harbouring genes encoding scFv was digested with NcoI/NotI and after purification of the scFv DNA in is ligated into the NcoI/NotI cut pKBJ3 vector. Furthermore, a DI vector was constructed which enables expression of DI without a fused antibody fragment. The oligos 5'-CAT GGC CGG GGC-'3 and 5'-GGC CGC CCC GGC-'3 were annealed by mixing 1µM of each oligo, heating to 100°C and subsequently cooling to 4°C. The annealed oligo was ligated into the NcoI/NotI cut pKBJ3 vector

4.4 Expression of scFvs and antibody purification

4.4.1 Production of antibodies

Hybridoma and CHO cells producing the antibodies A51-B/A6 (murine IgAκ), A46-B/B10 (murine IgMκ), A70-C/C8 (murine IgMκ), A70-A/A9 (murine IgG1κ), or cIgG CC8 (chimeric IgGκ) were cultured in RPMI 1640.

4.4.2 Purification of antibodies

All supernatants were prior to chromatography centrifuged for 30min at 4000g and passed through a 0.22µm filter to remove cells and cell debris. Prepared supernatants of IgG A70-A/A9 and cIgG CC8 were loaded on a 5ml protein A sepharose 4 Fast Flow column (Amersham Biosciences Europe GmbH, Freiburg, Germany). After washing to base line with approximately 20ml of buffer A (20mM phosphate, 150mM NaCl, pH 7.4) elution was performed with buffer B (20mM citrate, 150mM NaCl, pH 4).

The IgMs and the IgA were also prepared with centrifugation and filtration and subsequently added NaCl to 500mM. The supernatants were loaded on a 5ml HiTrap NHS-activated column (Amersham Biosciences Europe GmbH, Freiburg, Germany) with immobilised rabbit anti-mouse Ig (immobilised according to manufactures instructions). The column was washed with (10mM phosphate, 500mM NaCl, pH 7.2) and eluted with (50mM citric acid, 150mM NaCl, pH 2.2) into 1ml fractions neutralised with 200µl (2M phosphate, pH 7.2). Final preparation of pure antibody preparations was done though rebuffering to PBS (50mM phosphate, 300mM NaCl, pH 8.0) by centricon spin-tubes (Millipore GmbH, Schwalbach, Germany).

4.4.3 Production and isolation of antibodies from Prolifix supplemented media

Hybridoma cells producing the antibodies PankoMab (murine IgG1κ) or A70-C/C8 (murine IgM, κ) were cultured in RPMI 1640 supplemented with 5% Prolifix (Bio Media, Aachen, Germany). Supernatants containing the antibodies were centrifuged for 30 min at 4000g and passed through a 0.22µm filter to remove cells and cell debris. Antibodies were isolated, concentrated and the buffer exchanged to PBS by ultrafiltration using Centriprep YM-50 (Millipore, Eschborn, Germany) with a 50kDa molecular cut off membrane and centrifugation at 1500g.

To precipitate the DNA 10µl of 1M $MnCl_2$ or 1M $CaCl_2$ were added to 0.5 ml of ultrafiltrated antibody solution (pre-DNA-precipitation sample), briefly vortexed and centrifuged for 20 min at 16000g to allow sedimentation of a white pellet. The supernatant was carefully transferred to a new tube (post-DNA-precipitation sample). The pellet was resolved in 500µl 500mM EDTA (pellet sample). Aliquots of 16µl antibody pre-DNA-precipitation, 16µl post-DNA-precipitation, both added 16µl 500mM EDTA, 16µl resolved pellet added 16µl H_2O, and 5µl 100bp marker (100bp Ladder BioRad, München, Germany) were run on a 1.5% agarose gel and visualised by staining with ethidium bromide.

Antibody purity was estimated from UV absorption, measured at 260 and 280nm. The quotient A_{260}/A_{280} of 0.6 or below indicates a pure protein preparation. In SDS-PAGE analysis 10µl and 5µl β-mercaptoethanol-reduced samples, pre- and post-DNA-precipitation, were loaded on a 10% polyacrylamide gel. Subsequently the gel was stained with Serva Blue W for protein detection and the band intensities were compared.

4.4.4 Expression and harvesting of scFv and scFv-DI

Briefly, 50µl electrocompetent *E. coli* TG-1 were transformed with pHEN2 or pKBJ3 plasmid encoding a scFv. After electroporating the cells with the desired plasmid overnight cultures (2xTY media, 0.1mg/ml ampicillin) were incubated at 37°C. For each liter of 2xTY media, 0.1g/l ampicillin 10ml overnight culture is added and the suspension incubated at 37°C. At approximately A_{600} ~ 0.8 expressions is induced by adding IPTG to 1mM, followed by incubation for 4h at 30°C. Cell pellet per liter culture was resuspended in 40ml cold TES (30mM Tris, pH 8, 1mM EDTA, 20% Sucrose) buffer and incubated at 4°C for 20min with rotation; whereafter an equal volume of 5mM $MgSO_4$ is added. Finally, the TES fraction was cleared by centrifugation (4600rpm for 2h) and dialysed overnight against phosphate buffer (50mM phosphate, 300mM NaCl, pH 8.0).

4.4.5 Purification of scFv and scFv-DI

The scFvs and scFv-DIs were purified from the TES fractions by IMAC applying an open column with 5ml Chelating SepharoseTM Fast Flow (Amersham Biosciences) loaded with $NiSO_4$. After loading of the dialysed TES fractions by gravity flow, the column was washed with 50ml phosphate buffer (50mM phosphate, 300mM NaCl, 50mM imidazole, pH 8.0) and 10ml phosphate buffer (50mM phosphate, 300mM NaCl, 70mM imidazole, pH 8.0). The protein was eluted with phosphate buffer (50mM phosphate, 300mM NaCl, 300mM imidazole, pH 8.0), and dialysed against PBS overnight at 4°C.

The IMAC purified scFvs and scFv-DIs were dialysed to (50mM Tris, 20mM NaCl, pH 8). The protein was loaded on an open column with 5ml DEAE-sephadex column (Sigma-Aldrich, Steinheim, Germany) by gravity flow. The column was washed with 50ml Tris buffer (50mM Tris, 80mM NaCl, pH 8) and eluted with Tris buffer (50mM Tris, 150mM NaCl, pH 8). Eluted protein was rebuffered into PBS using centricon spin-tubes (Millipore GmbH, Schwalbach, Germany).

4.5 Antibody characterisation assays

4.5.1 ELISA

ELISA plates (MaxiSorp 96 well, Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 50µl antigen (10µg/ml for carbohydrate antigens, 4µg/ml for protein, and 1µg/ml glycosylated peptide in PBS), and blocked with 400µl 2% BSA-PBS for at least 60min at RT. The primary reagents (antibodies) were added as cell culture supernatants, as 1:30 diluted sera, or as recommended by the manufactures. Secondary and tertiary reagents were applied as phage supernatants or as recommended by the manufactures. Washing was performed between each incubation step by filling wells with ~400µl PBS (submerging) and "slamming" it out. ELISA's were developed by adding 100µl TMB substrate (3´.3´.5´.5´.-tetramethylbenzidine in dimethyl sulfoxide) and incubating for 20min. Reactions were quenched with 50 µl 2.5N H_2SO_4, and readings taken at 450nm with 630nm as control.

Anti-idiotypic controls included TEPC183 or MOPC-21 as antibody isotype controls, polyclonal affinipure goat

anti-mouse IgM as a control for cross species Ig binders, and the blocked empty wells for binders to plastic or the blocking agent. True Ab2s were defined as binding to Ab1 with an absorbance higher than 0.15 after subtraction of the background values, and control antibodies with an absorbance of less than 20% of the binding to Ab1. For isotype binders the signals with Ab1 and isotype were requested to surpass an OD of 0.15 with any background below 50%. Shared antibody epitope binders were defined as binders of Ab1, isotype, and capture antibody, which did not belong to the previous groups. As non-specific binders were those defined binding to all wells including the control without immobilised antibody whereby it was not differentiated between those binding to the blocking agent or those with a stickyness to the plastic.

4.5.2 Periodate oxidation

Carbohydrate-dependent binding of the antibody was tested by means of mild periodate oxidation according to (Woodward, Young et al. 1985). The procedure was applied in some assays prior to immunocytochemistry or ELISA. In brief, wells of an ELISA plate with immobilised Histone H1 (Alexis biochemicals, Heidelberg, Germany) Lewis Y conjugate, or multitest slides with fixed cells were first incubated with 10mM $NaIO_4$ in 0.1M acetate buffer (pH 4.5) for 1h at RT in the dark. For control wells acetate buffer only was used. The wells were washed with PBS and treated with 50mM $NaBH_4$ in PBS for 30min. After a further wash with PBS, the usual ELISA or immunocytochemistry procedures, respectively, were followed.

4.5.3 Western blots

Phage or protein samples were run on 15% SDS-PAGE, and electroblotted on nitrocellulose membranes using a semi-dry blotting device (Biorad). Membranes were blocked overnight in 2% BSA-PBS at 4°C, and blots performed in 50ml disposable tubes. Incubations were performed in 10ml 1% BSA-PBS with antibodies anti-p3 (Mo Bi Tec) as recommended by the manufacturers or 1:10 dilution of A70-A/A9 cell culture supernatant in BSA-PBS. Secondary reagent was the anti-mouse/HRP conjugate, and the blots were developed using the chemiluminescence kit (SuperSignal, Pierce, USA).

4.5.4 Surface plasmon resonance

Measurements were performed on a BIAsensor machine (BIAcore2000, Pharmacia) in PBS (pH 7.0) with a flow rate of 5µl/min at 25°C. Carbohydrate-paa-biotin (Lectinity Holdings, Inc, www.lectinity.com) was immobilised on streptavidin Sensor Chip SA using the procedure according to BIAcore protocols. A high surface density of carbohydrates (400-900 RU) was immobilised. For analysis of the antibodies, sensorgrams at 8 different concentrations (1µM, 700nM, 600nM, 500nM, 300nM, 150nM, 100nM, and 50nM) were obtained. Dissociation was analysed by injection of PBS. Buffer for the regeneration of the chip was citric acid (pH 2.2). Kinetic parameters were determined using the BIAevaluation program version 3.1. To obtain a functional affinity of the antibody the sensorgrams were fitted to a Langmuir 1:1 model.

4.5.5 Cell staining

Multiwell slides (10 wells, Roth) were loaded with around $3x10^4$ cells and incubated overnight in a CO_2 incubator. The supernatant was removed, and the slides were dried. The cells were permeabilised by freezing and thawing the slides once, and fixed on the slide with 5% formaldehyde in PBS for 5min. If the cells were coloured with 4'6-diamidino-2-phenylindole (DAPI, Sigma) 10µg/ml was added to the formaldehyde in PBS. After washing the primary antibody was added and incubated for 1h. After thorough washing the secondary antibody Cy3-conjugated diluted according to manufactures instructions in 1% BSA-PBS was added and incubation continued for 30min. The slides were mounted with mowiol. Pictures were taken using a Zeiss Axioplan microscope (C. Zeiss, Jena, Germany) equipped with an Axiocam camera.

4.5.6 Flow cytometry

Antibody or Lectin staining: About $3x10^5$ cells were incubated at 4°C for 1.5h with primary mAb or with the biotinylated lectin UEA I, followed by the secondary Cy2- or Cy3-conjugated antibody diluted as recommended by the manufacturer, or Cy3-streptavidin diluted 1:300 in PBS, respectively for 30min. at 4°C and washed again. Resuspended cells were examined with a Coulter Epics apparatus (Beckman Coulter, Krefeld, Germany).

For competition experiments the same setup as for staining was used. The amount of test antibody A70-A/A9 (or A70-C/C8) was kept constant (supernatants 1:30 diluted), and various amounts of competitor (A70-C/C8 or A70-A/A9) were added from 0.1-30 times in excess. Reversal competition was evaluated positive if both competitions could inhibit binding below 60%.

Quantitative analyses were carried out using the Expo32 software (Beckman Coulter).

4.5.7 Inhibition analysis

ELISA: Asialoglycophorin was immobilised onto 96-well plates at 100ng or 25ng per well in PBS. Alternatively, 5ng/well of biotinylated MUC1-Tn glycopeptide was immobilized in PBS on streptavidin coated plates (BioTez, Berlin, Germany) for 20min. Plates were washed with PBS and blocked with 30% FCS/RPMI for 2h at RT. Hybridoma supernatants of A78-G/A7 (1:5 and 1:20) or A76-A/C7 (1:60 and 1:160) were preincubated with various amounts of scFv-phages (10^{10} to 10^{12} phages of which approximately 1% carry scFv) in 10% FCS/RPMI/0.1% Tween20 for 1h at RT. Subsequently, 50µl/well were transferred to the antigen-coated plates for 1.5h at RT. After washing 4 times with TPBS, the plate was incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (P260; Dako, Hamburg, Germany) (1:2000)(in 10% FCS/RPMI/0.1% Tween20) for 1h at RT. Plates were washed and further processed as described above.

Immunocytochemistry: Multitest slides with cells from a TF-carrying subline of KG-1 (human acute myeloic leukaemia) were prepared and fixed with formaldehyde as described above. The KG-1 subline was selected for TF-carrying cells using magnetic beads coated with A78-G/A7. It constitutively expresses TF on more than 90% of its cells. The cell line was cultured in RPMI 1640 medium supplemented with 10% FCS. A78-G/A7

supernatant (1:20) was preincubated with 10^{12} phages, of which approximately 1% carry scFv, in 10% FCS/RPMI/0.1%Tween20 for 1 h, and subsequently added to the cells for 1.5 h. FITC-labelled goat anti-mouse immunoglobulin (1:200 in PBS, SIFIN, Berlin, Germany) was used as secondary reagent. Slides were mounted with Nairn buffer containing p-phenylenediamine to prevent fading and to obtain a slightly brownish fluorescent counterstaining of nuclei, and examined with a Zeiss (Jena, Germany) Axiophot fluorescence microscope. All steps were performed at RT.

Cell binding: Maxisorp plate (Nunc) was coated over night at 4°C with 50µl/well 5µg/ml A70-A/A9 in PBS. Subsequently it was washed once with PBS/Tween and blocked for 1h with 2% BSA/PBS and washed 2x with PBS/Tween. NM-F9 cells were labelled with Na-chromat (^{51}Cr, Amersham) by growing/incubation of cells for 2h, 37°C in the presence of 2MBq/ml cell growth medium (RPMI + 10%fcs). To remove unbound ^{51}Cr the cells were washed 2x with PBS and resuspended in cell growth medium. The assay was performed by addition of inhibitor, Human histone H1.2 (Alexis Biochemicals, Heidelberg, Germany) or Lewis b (Sigma-Aldrich, Steinheim, Germany) in a maximum of 50µl PBS and incubated for 30min at 4°C followed by addition of $1x10^5$ labelled cells in 10µl and incubation for 30min at 4°C. To remove unbound cells the wells were washed twice with PBS (100µl each), bound cells are lysed by addition of 100µl 0.1M NaOH and quantified by radioactivity measurement. Wells without inhibitor served as control. All tests were performed in triplicates.

IC50s are estimated from inhibition curves and compared.

4.5.8 Saturation transfer difference nuclear magnetic resonance

All NMR experiments were perfomed on a Bruker DRX600 spectrometer using an inverse triple resonance probe equipped with self-shielded three-axis gradients. NMR samples were prepared by resolving the lyophilised sample in 500µl 99.9% D_2O. A second lyophilisation was made to remove as much H_2O as possible and the sample was resolved in 500µl 99.99% D_2O. The molar ratio of carbohydrate:binding site was for all combinations of carbohydrate and antibody 100:1. All measurements were performed at 298K.

Several homonuclear and heteronuclear spectra were recorded for the assignment of carbohydrate resonances: A DQF-COSY was recorded using 8 scans, 2048 x 512 complex data points points and 10000 and 5555Hz spectral width in F2 and F1, respectively. Four TOCSY spectra were recorded with the same parameters and using mixing times of 16, 32, 64 and 112msec. An HMQC was recorded using 64 scans and 512 x 512 complex points and 10000 and 20833Hz in F2 and F1, respectively. An HMQC-COSY and three HMQC-TOCSYs were recorded with identical parameters and 96 scans, the three mixing times employed were 32, 64 and 112msec.

The study of carbohydrate-antibody binding was performed using the STD-technique. To remove signals from the antibody, a $T_{1\rho}$-filter of 30msec was implemented. The irradiation was performed using a train of shaped pulses with a total length of 2 seconds, a gaussian shape was used. The spectra were typically recorded using 4k complex points and 128 scans (Mayer and Meyer 2001).

Non-conjugated oligosaccharides containing a spacer as protective group $O\text{-}(CH_2)_3\text{-}NHCOCF_3$ was purchased at Lectinity Holdings, Inc (www.lectinity.com).

Assignment of signals were performed using the program Sparky (www.cgl.ucsf.edu/home/sparky/ (Goddard and Kneller)) and absolute integrals were calculated using the program MestRe-C (www.mestrec.com/MestRe-C.html).

4.6 Immunology assay

4.6.1 Mouse immunisation

The immunological properties of the scFvs and DI were examined in 10-12 weeks old Balb/C female mice, which were immunized twice with: L36 (50µg), L36 (50µg)+ Incomplete Freund's Adjuvant (IFA), L36-DI (50µg), PACA17 (50µg), PACA17 (50µg) + IFA, PACA17-DI (75µg), PACA17 + DI (50µg + 25µg), and DI alone (25µg). Each group consisted of three identically treated mice. Injections were given intraperitoneal (i.p.) at Day 0 and Day 14, and blood samples were collected at day –1 and day 28. Immunizations were performed by EPO GmbH, Berlin, Germany.

4.6.2 IgG1 and IgG2a antibody immune responses and sera specificity

The immunoassays were performed in Maxi-sorp™ plates (Nunc, Roskilde, Denmark) coated overnight at 4°C with 250µg/well scFv in PBS. After washing three times in PBS and blocking for 30min RT with 2% BSA-PBS, diluted sera in 2% BSA-PBS were added to the wells. After 3 washes the IgG subclasses were detected by incubation with 1:1000 dilution of polyclonal Rabbit IgG1 and IgG2a (Abcam, Cambridge,UK), respectively. After additional washing, a POD-conjugated goat anti-rabbit antibody (DAKO, Glostrup, Denmark) was used for detection. The ELISAs were developed using TMB-substrate, and subsequently quenched with 2.5M H_2SO_4. Absorbance of light with wavelength between 450nm and 650nm were measured.