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A proteomic approach to tumour target identification using phage display, affinity purification and mass spectrometry

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Abstract

Tumour-associated cell surface markers are potential targets for antibody-based therapies. We have obtained a panel of myeloid cell binding single chain variable fragments (scFv) by applying phage display selection on myeloid cell lines followed by a selection round on freshly isolated acute myeloid leukaemia (AML) blasts using flow cytometry. To identify the target antigens, the scFv were recloned and expressed in an IgG_1 format and tested for their ability to immunoprecipitate cell surface proteins. The IgGs that reacted with distinct cell membrane extractable proteins were used in large-scale affinity purification of the target antigen followed by mass-spectrometry-based identification. Well-characterised cell surface antigens, such as leukocyte antigen-related receptor protein tyrosine phosphatase (LAR PTP) and activated leukocyte adhesion molecule (ALCAM) in addition to several unknown proteins, like ATAD3A, were identified. These experiments demonstrate that phage antibody selection in combination with affinity chromatography and mass spectrometry can be exploited successfully to identify novel antibody target molecules on malignant cells.

Keywords: Phage display; Tumour antigen; Single chain Fv; AML; Myeloid; LAR PTP; ALCAM; ATAD3A

1. Introduction

The treatment of cancer by antibody therapy is rapidly becoming established in clinical practice. Currently, there are eight Food and Drug Administration (FDA) approved monoclonal antibodies for oncology applications, while an additional seven are in late-stage clinical development [1]. However, application of general antibody therapy is hampered by the lack of tumour-associated markers. Therefore, the identification of novel targets is a crucial step in designing and developing antibody-based therapies.

Antibody phage display is one of the technologies that can lead to the identification of novel target molecules. Several research groups have used antibody phage display selection strategies to isolate antibodies that bind to surface markers on tumour cells. This has resulted in phage antibodies directed against human melanoma cells [2–5], human lung carcinoma [6] and colorectal carcinoma cells [7,8]. However, the transition from the selection of cell-specific phage antibodies to the identification of the cellular target is still a major hurdle. For the identification of antigens, a genomic approach using cDNA expression cloning and a proteomic approach using affinity chromatography and mass spectrometry can be employed.

In this study, phage antibody selections were performed on myeloid cells resulting in the isolation of

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a panel of myeloid cell binding antibodies. To identify the target antigens, affinity purification followed by one-dimensional electrophoresis and mass spectrometry (MS)-analysis was used. The resulting antigen panel consists of several well-characterised proteins, leucocyte antigen-related receptor protein tyrosine phosphatase (LAR PTP), activated leucocyte adhesion molecule (ALCAM) and nicotinamide nucleotide adenylyltransferase, in addition to several hypothetical proteins. Restrictive expression of a selection of these target antigens on acute myeloid leukaemia (AML) was confirmed by fluorescence activated cell sorting (FACS) analysis with phage antibodies. Together, these results demonstrate the power of phage display in combination with affinity purification and MS for the identification of novel cell type-specific cell surface receptors.

2. Materials and methods

2.1. Cells and antibodies

All cell lines used were derived from the American Type Culture Collection (ATCC). NB4, HL60 and K562 were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat inactivated foetal bovine serum (FBS-HI) and 2 mM L-glutamine. HEK293T was cultured in Dulbeccos modified eagle medium (DMEM) supplemented with 10% FBS-HI and 0.4 mM L-glutamine. HEp-2 was cultured in DMEM supplemented with 10% FBS-HI and 2 mM L-glutamine. The colon adeno carcinoma cell line LS174T was cultured in DMEM supplemented with 10% FBS-HI.

Peripheral blood buffy coats were obtained from the Sanquin Blood Supply Foundation. Leukaemia cells were obtained from peripheral blood of newly diagnosed AML patients after informed consent and classified according to the French–American–British (FAB) classification.

Monoclonal antibodies used in this study: CD16-fluorescein isothiocyanate (FITC), CD19-APC, CD32-FITC, and CD33-phycoerythrin (PE) were obtained from Pharmingen, CD14-FITC, CD3-FITC, CD33-APC, CD34-PE and CD45-PerCP were from Becton–Dickinson and monoclonal anti-myc antibody (9E10) was from Santa Cruz.

2.2. Flow cytometry using scFv-phage

Reactivity profiles of phage antibodies were analysed using FACS analysis; essentially as described before in [9], except that biotinylated anti-M13 mAb (Roche) followed by streptavidin-PE (Caltag) was used to detect the cell bound phages.

2.3. Phage selections

Phage antibodies were selected using a semi-synthetic antibody phage display library [9] rescued with the CT helper phage [10]. An aliquot of the phage library ($\sim 1.5 \times 10^{13}$ colony forming units (cfu)) was blocked for 30 min on ice in RPMI 1640 containing 10% FCS (RPMI/FCS). Subsequently, the library was subtracted three times with 2.3×10^8 peripheral blood cells (PBL) in 10 ml RPMI/FCS for 2 h at 4 °C, to deplete phage antibodies binding antigens on non-tumorigenic cells. After centrifugation to remove the absorber cells, the phage-containing supernatant was incubated in parallel with 4×10^6 cells of HL60, K562 and NB4 for 2.5 h at 4 °C. After removal of unbound phages, cell bound phages were eluted and rescued as described in [9,10]. A second selection round on primary AML tumour cells (CD34+ blasts, FAB M0) was performed by incubating blocked phages for 2.5 h at 4 °C with 5×10^6 CD34PE labelled AML cells. After washing in RPMI/FCS, CD34+ cells with attached phages were sorted on a FACSVantage flow cytometer [9]. Attached phages were eluted, rescued and monoclonal phage preparations were generated for binding analysis on PBL, cell lines and AML tumour samples as described previously in [10]. Binding clones were sequenced to identity unique clones as described previously in [11].

2.4. Generation of human IgG_1 antibodies

The engineering and production of the human IgG_1 monoclonal antibodies was performed as described in detail by Boel and colleagues in [12]. The variable regions of the scFv were recloned in separate vectors for IgG_1 heavy and light chain expression. VH and VL regions from each scFv were polymerase chain reaction (PCR) amplified using primers to append restriction sites and restore complete human frameworks. IgG_1 were produced as described previously in [12] and purified on protein-A columns followed by buffer exchanging in PBS over size-exclusion columns (Amersham Biosciences).

2.5. Biotinylation of cell surface molecules and immunoprecipitation

Small-scale immunoprecipitation procedures were performed of each antibody/antigen combination to determine the optimal conditions in large-scale affinity purification.The cell surfaces of 10^8 human LS174T, HEp-2 and NB4 were biotinylated for 1 h at room temperature (RT) with 2 mg Sulfo-NHS-LC-LC-biotin per 10^8 cells (Pierce) in physiological buffer (0.2 M phosphate buffer containing 0.12 M NaCl, pH 7.4 (PBN). After blocking of free biotin with 10 mM glycine (Gibco) in PBN, the cells were lysed at a concentration

of 3×10^7 cells/ml in three different lysis buffers containing protease inhibitors (Roche) for 30 min at 4 °C to establish the optimal lysis buffer for every antibody–antigen combination. The detergents tested varied from the mild non-denaturing Triton X-100 buffer (1% Triton X-100 (Serva), 150 mM NaCl, 50 mM Tris, pH 7.4), to more denaturing buffers that in addition included 0.1% of the bile salt sodium desoxychelate (1% Triton X-100 (Serva), 150 mM NaCl, 50 mM Tris, pH 7.4) and sodium dodecyl sulphate (SDS) that are both present in RIPA buffer (1% Triton X-100, 0.5% DOC, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4). After removal of insoluble material by centrifugation, the biotinylated lysates were pre-cleared with protein A beads for 2 h at 4 °C at 5 rotations per minute (rpm) in an end-over-end rotor. Selected and control IgG_1 antibodies (4 μg) were coupled to protein A beads. The negative control CR2428 is directed against a bacterial epitope not expressed on eukaryotic cells and the positive control CR2300 is directed against the cell surface marker, CD46. The pre-cleared lysates (an equivalent of 3×10^7 cells) were incubated with the antibody-coated protein A beads for 2 h at 4 °C at 5 rpm in an end-over-end rotor. The beads carrying the immune complexes were washed 3 times for 5 min with lysis buffer before analysis on SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot.

Except for CR2361, all antibodies performed best in Triton X-100 lysis buffer. To establish the final conditions for affinity purification, the optimal wash and elution conditions for each antibody/antigen combination were determined in the small-scale immunoprecipitation assays. Therefore, immunoprecipitations were performed in the optimal lysis buffer and beads carrying the immune complexes were subjected to washes with increasing stringency by raising the NaCl concentration of the buffer to 1 M NaCl and by using lysis buffers containing detergents with increasing harshness (Triton X-100, RIPA). Subsequently, elution conditions of the proteins from the beads were tested using low (glycine pH 2.7) or high (lysine, pH 11) pH elution.

2.6. SDS/PAGE and immunodetection

Immune complexes were analysed by electrophoresis through 4–12% Bis Tris NuPAGE Novex gels (Invitrogen) under reducing or non-reducing conditions. After electrophoresis, the proteins were electroblotted on poly vinylidene difluoride (PVDF) membranes (Schleicher and Schuell) and the membranes were blocked for 1 h at RT with 3% bovine serum albumin (BSA) (Sigma) in PBS (PBS–BSA) for the detection of biotinylated proteins or with 5% non-fat milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.3% Tween 20) for immunoblot detection. Subsequently, the blots were incubated for 1 h at RT with horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech), diluted 1:5000 in PBS–BSA, horseradish peroxidase-conjugated mouse anti-myc (Roche), diluted 1:10,000, mouse anti-LAR (Chemicon), diluted 1:250 or mouse anti-ALCAM (Serotec) diluted 1:100 in 0.5% milk in TBST. After washing three times with TBST, the membranes incubated with non-conjugated IgG were subsequently incubated with horseradish peroxidase coupled rabbit anti-mouse-Ig (1:5000 dilution) for an additional hour at RT. After three washes with TBST buffer, bound peroxidase was detected by enhanced chemiluminescence as described by the manufacturer (Amersham Pharmacia Biotech).

2.7. Preparation of cell extracts for large-scale affinity purification

Lysates were prepared from $4.5–6 \times 10^9$ NB4, HEp-2 and LS174T cells. The adherent cells in this panel were grown to 70% confluency and then detached by trypsinisation. Cell pellets were collected by centrifugation and washed twice in PBS. The cells were then suspended in 100–200 ml Triton X-100 lysis buffer except for the NB4 cells, which were suspended in RIPA lysis buffer, followed by 30–60 min incubation on ice. After removal of insoluble material by centrifugation (at 20,000*g*), the cell lysates were passed through a 0.22-μM filter and stored on ice until further use.

2.8. Large scale affinity purification

1–2 mg of the selected IgG_1s were passed on a 100-kDa Ultracentrifugal device (Amicon) to remove incompletely formed IgG fragments. Next, the IgG_1s were coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's specifications. The individual cell lysates were pre-cleared for 4 h at 4 °C with 60 ml blocked CNBr-activated Sepharose CL-4B beads, followed by a pre-clearing step for 4 h at 4 °C with 5 ml of CNBr-activated beads to which human control IgG_1 was coupled (1 mg IgG_1/ml, Sigma). Next, the individual lysates were passed through a 0.22-μM filter to remove insoluble material. The negative control CR2428 column and one of the affinity columns were connected in series to a ÄKTA FPLC 900 and equilibrated with lysis buffer. The lysates were applied to the columns at 2 ml/min; columns were washed with lysis buffer and, if required, washed with a salt gradient up to 1 M NaCl, followed by a TX-100 lysis buffer wash. The columns were eluted with 0.1 M glycine, 150 mM NaCl, 1% Triton X-100, pH 2.7, except for the CR2401 column that was eluted with 0.1 M L-lysine, 150 mM NaCl, 1% Triton X-100, pH 11. The eluted fractions were neutralised using Tris base or acetate, and analysed on silver-stained SDS-PAGE gels (Invitrogen). Proteins present in the eluting profiles that

corresponded in size to the sizes of proteins obtained by the immunoprecipitations were cut out from the gels with a sharp razor and sent off for commercial microsequencing by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF)MS (TOPlab Martinsried, Germany) or (MALDI-TOF)MS and nano-electrospray ionisation tandem MS (nanoESI-MS-MS) (MDS, Odense, Denmark).

2.9. cDNA cloning of ATAD3A protein, construction of myc-tagged clones and transfection

RNA was isolated from 2×10^7 NB4 cells using the Nucleotrap mRNA mini-purification Kit according to the manufacturer's instructions (Becton–Dickinson) cDNA was synthesised from 1 μg of total mRNA with MMLV reverse transcriptase using the 'Advantage RT for PCR Kit' (Clontech) according to the recommended instructions. Five microlitres of the 20 μl cDNA reaction was used as a template in the following PCR to amplify a full-length *ATAD3A* transcript. In a total volume of 50 μl, a PCR was performed with primers 5′-CCCCACTTCTGTCTAGTCC-3′ and 5′-CCGTGAGCTGTGGATCTCC-3′ using *Pfu* polymerase (Promega). After 3 min initial denaturation at 94 °C, 25 cycles of 94 °C for 40 s, 57 °C for 1 min, 72 °C for 2 min were performed followed by a final extension of 5 min at 72 °C. The PCR product was analysed by agarose gel electrophoresis and the expected 1800 bp fragment was isolated with the Min-elute gel extraction Kit (Qiagen). The fragment was cloned in the pCR4TOPO vector (Invitrogen) and transformed into DH5α. The resulting clone was verified by sequence analysis and subsequently digested with *Eco*RI and cloned in the corresponding sites of pcDNA3.1zeo (Invitrogen). To simplify the detection of ATAD3A protein in the subsequent transfection experiments, *ATAD3A* was fused with a myc tag at the 5-prime and 3-prime end by PCR (using the *ATAD3A*-pCR4TOPO construct as a template). For the 5′myc construct, the following primers were designed; forward primer 5′-CGGGATCCAGCATGGAACAAAAACTTATTTCTGAAGAAGATCTGTCGTGGCTCTTCGGCATTAACAAG-3′, reverse primer 5′-CGGAATTCGACTCAGGATGGGGAAGGC-3′. For the 3′myc construct, the primers were constructed in such a way that *ATAD3A* became in frame with the myc tag in pcDNA3mycA. Forward primer, 5′-CGGGATCCTGCGAGCATGTCGTGGC-3′. Reverse primer, 5′-GCTCTAGAGGATGGGGAAGGCTCG-3. PCRs were performed using *Pfu* polymerase and the resulting fragment of the 5′myc tag was cloned *Bam*HI/*Eco*RI in the pcDNA3.1zeo vector (Invitrogen) resulting in the ^{myc}*ATAD3A* construct, whereas the resulting fragment for the 3′myc tag was cloned *Bam*HI/*Xba*I in pcDNA3.1/hismycA (Invitrogen) resulting in the *ATAD3A*^{myc} construct'. *ATAD3A*, *ATAD3A*^{myc} and ^{myc}*ATAD3A* were transfected into HEK293T cells using Fugene reagent (Roche) according to the manufacturer's instructions. Seventy two hours after transfection, cells were harvested and analysed by flow cytometry or lysed in 1% Triton X-100 in PBN followed by biotinylation as described above.

2.10. Flow cytometry of primary AML and PBL

Peripheral blood cells were obtained from healthy donors and AML patients. Mononuclear cells were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) differential centrifugation followed by lysis of the remaining red blood cells with ammonium chloride. Cells were cryopreserved until use. Upon thawing, 2×10^5 cells were blocked with 50 μg/ml rabbit IgG (Dako) for 20 min on ice. Phage staining was performed according to the phage staining protocol described above. Subsequently, tumour cells were incubated with the monoclonal antibodies CD33-APC and CD45-PerCP, whereas healthy cells were incubated with CD3-FITC, CD14-FITC, CD16-FITC, CD19-APC and CD33-APC for 30 min on ice. Cells were washed twice with PBS/1% BSA and tumour cells were stained for AnnexinV-FITC in addition, (Becton–Dickinson) according to the manufacturers conditions, to exclude the dead cells. Cells were analysed on a FACSCalibur (Becton and Dickinson) using Cell Quest software. For final analysis of the tumour cells, blast cells were gated based on low side-scatter versus CD45 expression. A sample was considered positive if more than 20% of these cells expressed the antigen of interest. Healthy PBLs were gated based on cell population-specific surface markers in order to analyse the antigen expression per individual cell population.

3. Results

3.1. Selection of phage antibodies on AML and myeloid cells

Antibody phage display was used to identify novel surface markers on myeloid cells. To select for antibodies preferentially binding to myeloid cells, a subtraction using healthy donor peripheral blood leucocytes was employed to deplete for antibodies binding molecules on these non-tumorigenic cells. After one round of selection on a panel of myeloid cell lines, a second selection round was performed on primary AML blasts representing the M0 stage of haematopoietic development. The selected phages were tested for their reactivity towards AML, myeloid cell lines and peripheral blood leucocytes. Subsequently, positive clones that did not react or showed a restricted reactivity with the peripheral blood leucocytes were subjected to sequence analysis, comparison

of sequences revealed that 27 unique antibodies were selected. From this panel of antibodies, 13 clones were selected for target identification on the basis of their broad AML recognition and their lack or restricted reactivity with healthy donor peripheral blood leucocytes.

To test the reactivity of the clones to a broader range of tumour cell lines, the phage antibodies were analysed by flow cytometry for binding to different myeloid cells (NB4, K562, THP-1) and trypsinised colon (LS174T), breast (MCF-7), cervix (HEp-2) and embryonic kidney (HEK 293T) cells. The cell line that showed the highest immunoreactivity with a particular phage antibody and was thus expressing the highest antigen density, was selected for the immunoprecipitation experiments. Interestingly, binding of several phage antibodies was not restricted to the cell compartment to which they were originally selected. Fig. 1 shows an example of the characteristic binding profile of three antibodies against one myeloid, cervix, colon and kidney cell line each. Note that each phage antibody had a distinct binding profile amongst the cell lines tested. As an example, antibody CR2361 preferentially bounds to NB4 cells, while antibody CR2407 bound to all of the cell lines tested.

3.2. Immunoprecipitation of the target antigen

Antigens corresponding to the selected antibodies were characterised by immunoprecipitation experiments. To increase the chance of success in these experiments, a higher avidity antibody format was created by first recloning the scFv into complete IgG_1s. Next, cell surfaces were labelled with biotin, extracted in detergent-containing buffer and subjected to immunoprecipitation with the selected antibodies. As a positive control antibody, clone CR3300 directed against CD46, a cell surface receptor present on every nucleated human cell

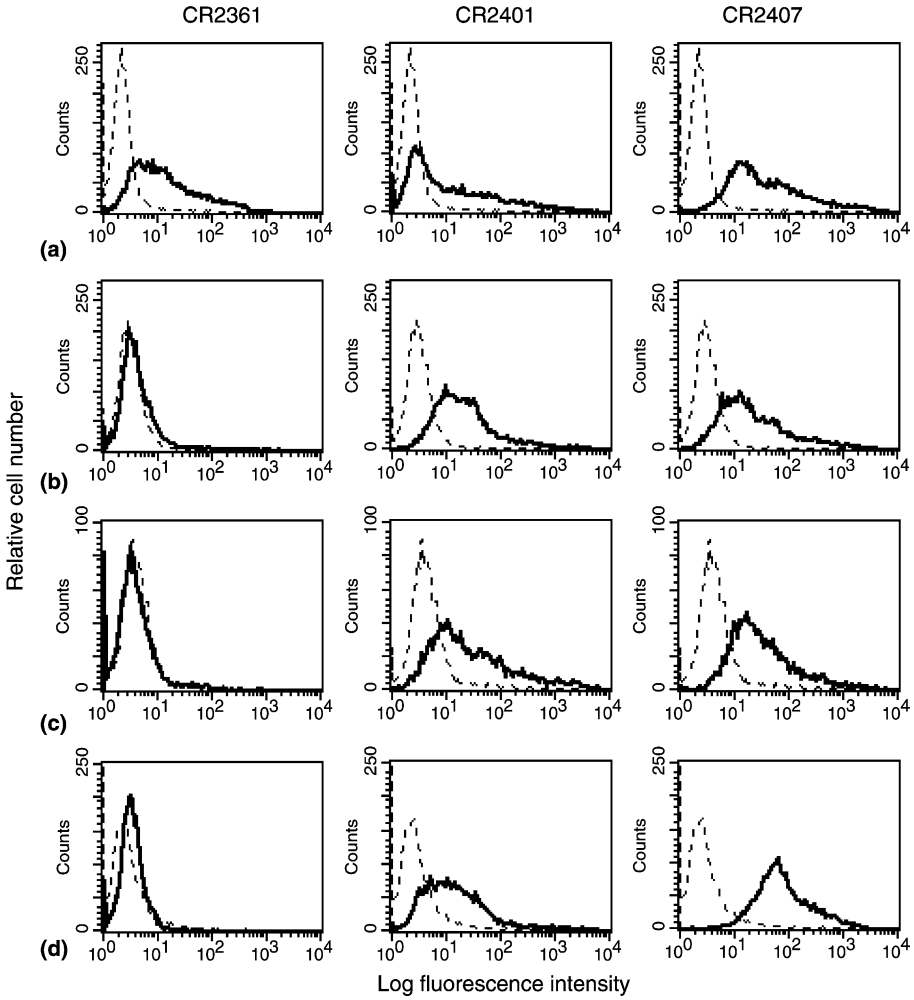

Fig. 1. Flow cytometric analysis of four phage antibodies binding to different tumour cell lines (a) myeloid NB4 cells, (b) HEp-2, (c) colon carcinoma cells, LS174T (D) HEK293T human embryonic kidney cells. Different tumour cell lines were incubated with the phage antibodies, CR2361, CR2401 and CR2407 (solid line) or a human IgG_1 control antibody (dotted line) followed by an incubation with a biotinylated anti-M13 antibody and phycoerythrin (PE)-labelled streptavidin. Binding of the phage antibodies was visualised by flow cytometry. The *Y*-axis represents the relative cell number, while the *X*-axis represents the log fluorescence intensity.

was used. The biotinylated proteins were analysed by SDS-PAGE and immunoblotting using a streptavidin-horse radish peroxidase conjugate.

Fig. 2 shows an example of SDS-PAGE analysed immunoprecipitations on biotinylated cell lysates with four of the selected antibodies. For each immunoprecipitation, major biotinylated bands appeared, ranging in size from 20 to 160 kDa. In the CR2361 immunoprecipitation, four bands running at 30, 40, 70 and 140 kDa were detected (Fig. 2(a)), two bands at 45 kDa and 160 kDa were visible from the CR2401 immunoprecipitation (Fig. 2(b)) and the CR2407 immunoprecipitation showed one broad band of 80–90 kDa and one sharp band of 160 kDa (Fig. 2(c)). None of these bands were present in immunoprecipitations performed with negative control IgG or human IgG directed against CD46. The biotinylated bands that were identified after immunoprecipitation with anti-CD46 antibody CR2300, show a different pattern for each cell lysate used. This can be explained by the presence of different isoforms of CD46, a glycoprotein that can be expressed in a single, double and hyperglycosylated form [13]. Of the 13 IgGs analysed, three failed to precipitate a distinct pattern of biotinylated proteins, in spite of the fact that different cell lines and solubilisation techniques were used (Table 1). Furthermore, some antibodies almost certainly precipitated the same antigen since identical patterns of biotinylated proteins could be observed on gel (Table 1). Of the antibodies that appeared to recognise the same antigen, those with the most optimal immunoprecipitation performance were pursued for further study.

3.3. Purification of the target antigen

The purification procedures for the target antigens of the 8 remaining antibodies were optimised by small-scale immunoprecipitation experiments with biotinylated lysates. Fig. 3 presents a non-reducing SDS-PAGE profile of the proteins eluting from the CR2361 column and shows that proteins of 30, 40, 70 and 140 kDa (indicated by the arrow) were released from the column. This protein profile corresponds exactly to the biotinylation profile obtained with the small-scale immunoprecipitation experiments (see Fig. 2(a)). Large-scale affinity chromatography with the other seven antibodies also resulted in the purification of proteins of the predicted size compared with the small-scale immunoprecipitations. The protein bands of interest were excised from the gels and sent out for commercial micro-sequencing by MALDI-TOF MS and/or nano-electrospray ionisation tandem MS (nanoESI-MS-MS), depending on the quantity of the purified protein.

3.4. Identification of the target antigen

Of the eight affinity purifications performed, identification was successful for proteins purified from six of them. Using these IgGs, a total of 12 proteins was purified from which 9 proteins were identified on the basis of MS analysis (Table 1). The identified proteins include two receptor proteins (LAR PTP and ALCAM), one cytoplasmic protein (nicotinamide nucleotide adenylyltransferase 1), two hypothetical proteins (CAD38982.1 and ATAD3A protein) and two proteins that were labelled as open reading frames (chr. 14 ORF145, chr. 17 ORF 1A). As shown in Fig. 3(b), a dozen peptides were identified from the 30, 40 and 70 kDa proteins purified via the CR2361 affinity column. Remarkably, all peptides matched with the same protein, the hypothetical ATAD3A protein.

To confirm the results obtained by our target identification approach, two receptor proteins and one hypothetical protein were selected for confirmation of the identification procedure. Protein purified via the CR2401 antibody was confirmed as being LAR PTP on Western blot using a specific monoclonal antibody (Fig. 4(a)). In the purified fraction, two additional bands of a slightly lower molecular weight also reacted with the anti-LAR PTP antibody and most likely represented

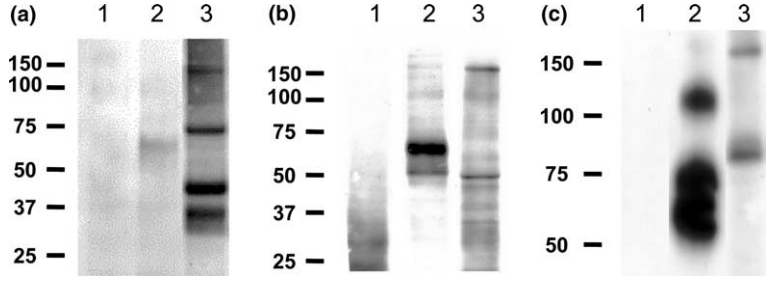

Fig. 2. Immunoprecipitation of biotinylated cell surface proteins with antibodies CR2361, CR2401 and CR2407. Immunoprecipitated proteins were analysed under non-reducing conditions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and probing with streptavidin coupled to horseradish peroxidase. (a) CR2361 on NB4 cell lysate, (b) CR2401 on LS174T cell lysate, (c) CR2407 on HEp-2 cell lysate. Proteins in the cell lysates were precipitated with a human IgG_1negative control antibody (lanes 1), a human IgG_1directed against CD46 (lanes 2) and human IgG_1 of the selected antibodies (lanes 3).

Table 1
Identification procedure and results

Antibody	Precipitated proteins (size, kDa)	Purified proteins (size, kDa)	MS identified protein (name)	Abbreviation	SwissProt/Trembl	Confirmation (method)
CR2365	–[a]					
CR2366	–[a]					
CR2395	–[a]					
CR2124	*CR2367 profile*					
CR2416	*CR2 414 profile*					
CR2062	35	35	Unsuccessful			
	55	55	Unsuccessful			
CR2414	85	85	Unsuccessful			
CR2128	30	30	Nicotinamide nucleotide adenylyltransferase 1	NMNt	gi|20070321	NA
	80	80	Unsuccessful			
	100	100	Unsuccessful			
CR2360	130	130	CAD38982.1		gi|21739918	NA
	100	100	chr. 17 ORF 1A		gi|20306882	NA
CR2361	30	30	ATAD3A protein	NA	gi|21620024	cDNA
	40	40	ATAD3A protein			
	70	70	ATAD3A protein			
	140	140	Unsuccessful			
CR2367	20	–[b]				
	40	–[b]				
	90	90	chr14 ORF145		gi|22748933	NA
CR2401	45	–[b]	Leucocyte antigen-related receptor protein tyrosine phosphatase	LAR PTP	gi|4506311	Western
	160	160				
CR2407	80–90	80–90	Activated leucocyte adhesion molecule	ALCAM	gi|3183975	Western
	160	160				

NA, non-applicable.
[a] No proteins were specifically immunoprecipitated.
[b] No proteins were purified.

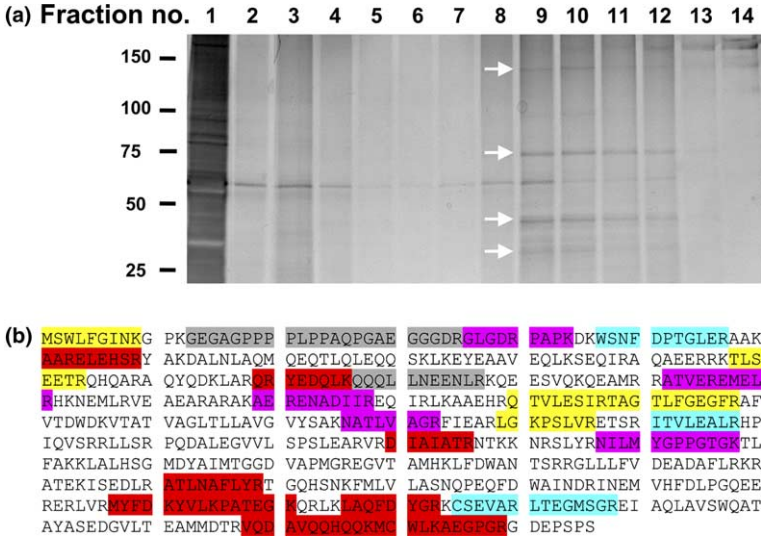

Fig. 3. (a) Purification of the CR2361 target antigen by large-scale immuno-affinity chromatography. NB4 cells were lysed and applied to the CR2361 affinity column. Following washes in a buffer that contains increasing NaCl concentration (fractions 2–7), proteins retained on the column were eluted by decreasing the buffer pH to 2.7 (fractions 9–13). Fractions eluted from the column were subjected to non-reducing SDS-PAGE and visualised using silver staining. The arrows indicate the 30, 40, 70 and 140 kDa target antigen, that were excised for mass spectroscopy. (b) Peptides matching ATAD3A protein identified by matrix-associated laser desorption/ionisation-time of flight (MALDI-TOF)/MS were indicated in different colours (yellow, peptides identified in the 30 kDa band; blue, peptides identified in the 40 kDa band; red, peptides identified in the 70 kDa band; pink, peptides identified in the 30 and 40 kDa bands; grey, peptides identified in the 30, 40 and 70 kDa bands). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

LAR PTP degradation products that could also be observed on the silver-stained gel of the eluted fractions (data not shown). The presence of ALCAM in the purified fraction of the CR2407 antibody was assessed by immunoblotting with an ALCAM-specific monoclonal antibody (Fig. 4(b)). In addition to the expected protein band of 80–90 kDa, a 55-kDa protein band could be detected. It has been reported recently that ALCAM can be cleaved by matrix metalloproteinase (MMP)-2 [14].

ATAD3A protein, which shares homology to the AAA-ATPase TOB3 was selected for further confirmation, since the ATAD3A protein identification was found with three proteins of different size that were purified with the CR2361 antibody. Full length human *ATAD3A* was generated by reverse transcriptase (RT)-PCR from mRNA extracted from NB4 cells. To simplify the detection of ATAD3A protein in the subsequent experiments, the cDNA was fused to the myc-tag peptide at the N and C termini. The cDNA was transfected into HEK293T cells and the expression of myc-tagged ATAD3A was studied by immunoprecipitation and flow cytometry analysis. Immunoblots with anti-myc demonstrated that both myc-tagged ATAD3A proteins could be immunoprecipitated from the cytoplasmic fraction by CR2361, but not by the control antibodies (Fig. 4(c)). Immunoprecipitations with biotinylated cell lysates revealed that the molecular weight of the cloned ATAD3A corresponded to the purified 75 kDa protein (data not shown). Both CR2361 and anti-myc antibodies failed to detect myc-tagged ATAD3A on transfected HEK293T cells, indicating that in contrast to the NB4 cells the protein was not expressed at the cell surface.

3.5. Expression of LAR PTP, ALCAM and ATAD3A protein on AML compared with normal peripheral blood leucocytes

The detection procedure identified targets selectively present on several tumour cell lines. To investigate whether the identified targets were specifically upregulated on different types of AML blasts compared with normal peripheral blood leucocytes, a panel of 28 AML samples, representing all stages of haematopoietic development, were analysed for LAR PTP, ALCAM and ATAD3A protein expression. For the FACS analysis, phage antibodies instead of IgGs were used, since phage antibodies in contrast to IgG molecules do not bind to the Fc receptors present on the AML blasts, which simplifies the analysis. In the analysis, different FAB types were studied and a gate was set to include only the $CD45^+$ cells with lymphoid-blast phenotype. To analyse the expression of the identified antigens in every individual cell population of normal donor peripheral blood leucocytes, the cells were subdivided into distinct populations on the basis of forward- and sideward-scatter properties and CD3, CD14, CD16 and CD19 expression [15]. Table 2 demonstrates that two of the targets, ALCAM and ATAD3A, were present on more than 20% of blast cells in most of the AML samples tested. 89% of the AML samples expressed ALCAM, whereas 77% of the AML samples expressed ATAD3A. Within peripheral blood the antibody directed against ALCAM solely recognised a subpopulation of dendritic cells (Table 2). The antibody directed against ATAD3A protein recognised half of the monocyte population and a subpopulation of dendritic cells, but no other lymphocytes. However, the expression of ATAD3A and ALCAM on monocytes and dendritic cells was, on average, 400-fold less than on the AML samples (data not shown).

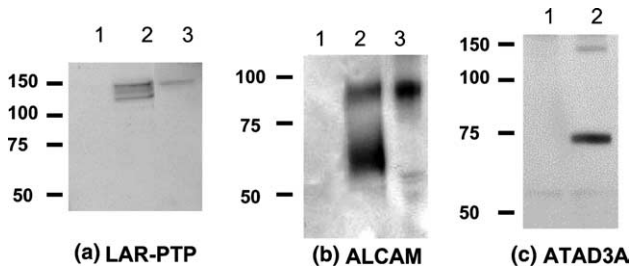

Fig. 4. Confirmation of the identification procedure of the target antigen. Immunoblot analysis using anti-LAR (a) and anti-ALCAM (b) antibodies: Lanes 1, eluted fraction from the control antibody column; lanes 2, eluted fraction from the relevant affinity column; lanes 3, total cell lysates. (c) Immunoblot using the anti-myc antibody on immunoprecipitations of cell lysates of HEK293T cells transfected with ^{myc}ATAD3A DNA. Lane 1, human IgG_1control antibody and lane 2, the CR2361 antibody. Results for $ATAD3A^{myc}$ were identical.

4. Discussion

We describe here the use of affinity purification in combination with MS to identify targets recognised by phage antibodies selected on AML and myeloid cell lines. Phage selection on whole cells or tissues often leads to the generation of numerous phage antibodies. In order to reduce the number of phage clones before embarking on labour-intensive target identification we used a defined strategy, as outlined in Fig. 5. The phage antibodies were first selected on the basis of their binding profile on a broad panel of cell lines and a restricted binding to healthy peripheral blood cells. Clones that passed this criterion were reformatted into IgGs and used in small-scale immunoprecipitation experiments to analyse whether the selected antibody bound to a membrane extractable protein. These antibodies were then used in large-scale affinity chromatography to purify the corresponding antigens. Purified proteins with the expected size were identified by MS analysis. Using this strategy, 7 target antigens could be identified that were purified with 6 antibodies. Three of the identified target proteins were selected for confirmation of the

Table 2
Expression of identified targets on primary acute myeloid leukaemia (AML) samples and *healthy peripheral* blood cells

Target	AML subtypes							
	M0	M1	M2	M3	M4	M5	Unclassified	% Positive on all AML
ALCAM	+	+	+	+	+	+	+	89 (25/28)
LAR PTP	+	–	–	+	–	–	-	29 (8/28)
ATAD3A	+	+	+	+	+	+	+	77 (20/26)
	Healthy blood							
	Monocytes	Granulocytes	B cells	T cells	Dendritic cells	Natural Killer cells	Erythryocytes	Platelets
ALCAM	–	–	–	–	S^2+	–	–	–
LAR PTP	–	–	–	–	–	–	–	–
ATAD3A	S^1+	–	–	–	S^2+	–	–	–

Expression of identified target antigens on AML and peripheral blood cells and platelets analysed by fluorescent activated cell sorting (FACS). A total of 28 AML samples (26 for ATAD3A) were analysed for cell surface expression of the antigen.
Cells within peripheral blood were subdivided into distinct populations on the basis of forward- and sideward-scatter properties and CD3, CD14/CD16 and CD19 expressions [15]. S^1+, 50% of the cells positive; S^2+, 40% of the cells positive. Mean fluorescence of *ATAD3A and ALCAM on monocytes and dendritic* cells in normal blood is, on average, 400-fold less compared with AML samples.

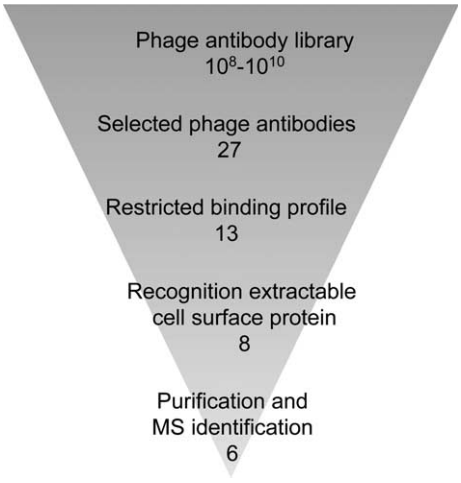

Fig. 5. Selection filter for target identification of phage antibodies. Filter one selects for cell-specific antigens using Mabstract™; filter two selects for antibodies that recognise a broad panel of AML and tumour cell lines, while not binding to healthy donor peripheral blood cells; filter three selects for antibodies that recognise membrane extractable antigens, that can subsequently be obtained via large-scale affinity purification; filter four finally leads to the identification of the protein via MS analysis.

identification by immunoblot and transfection experiments, all of these could be confirmed, thereby demonstrating the high success rate of this approach.

Recently, Gao and colleagues [16] demonstrated the identification of target antigens of tumour-specific internalising scFvs using immunoprecipitation with scFvs indirectly coupled to an affinity matrix. However, their approach is highly dependent on the density of the antigen at the cell surface and leads to the release of the scFv during the elution. Our affinity purification procedure is less dependent on the antigen density since large cell lysates can be processed. The effectivity of immunoprecipitations is also highly dependent on the extractability of the proteins from the membrane as some proteins that are directly connected to the cytoskeleton can only be extracted using harsh detergents that may affect the recognition of the epitope. Performing a small-scale immunoprecipitation using biotinylated cells will identify these targets so that they can be excluded for further analysis by affinity purification.

In our experiments often more than one protein is precipitated. One explanation is that the target forms a part of a heteromeric protein complex that is not dissociated during the solubilisation and/or purification procedure. Purification therefore results in multiple proteins being isolated, one of them containing the epitope. As an example, nicotinamide nucleotide adenylyltransferase 1 may represent a cytosolic enzyme that became co-purified with a non-identified membrane protein. Precipitation of multiple proteins may also be caused by the presence of isoforms of a single molecule, as appears to be the case for ATAD3A.

Another commonly used technique for the identification of antigens is expression cloning using cDNA libraries. In this method, cDNA libraries, expressed in either prokaryotes or eukaryotes, are probed with the antibodies. This approach does have several restrictions. As an example, antigens whose cell surface expression is dependent upon complex formation with other proteins will be difficult to identify via this method [17]. Furthermore, cell type-dependent differences in membrane protein trafficking have been demonstrated for various proteins [18,19]. This was illustrated by one of the identified targets in our panel, ATAD3A, which could not be expressed in HEK293T cells normally used for screening of cDNA expression libraries.

The antigens selected using our approach can be divided into previously identified tumour antigens and novel antigens. Antigens belonging to the first group are ALCAM and LAR PTP, antigens that are also expressed on healthy cells but that are overexpressed on tumour cells. ALCAM has been identified as an overex-

pressed marker on metastatic human melanoma cells and myeloma cells [20,21] and LAR PTP is overexpressed in thyroid carcinomas [22] and breast cancer [23]. More interesting, several novel proteins were detected; CAD 38982.1, chr.17 ORF 1A, chr 14 ORF 145 and ATAD3A protein have been submitted to databases by various genome analysing groups, but additional data on these proteins are not available. However, the selective expression of these molecules does warrant future experiments to investigate their significance. Initial ATAD3A expression data on AML presented in this paper show the potential of this molecule as an AML marker for diagnostic applications.

In conclusion, antibody phage display selection on tumour cells can be an efficient technology for the selection of cell type-specific antibodies. The output of the technology was previously hampered by difficulties experienced when identifying the antibody targets. Here, we show that the combination of phage display with affinity purification and MS is a powerful approach to identify novel target molecules that may find application in antibody therapy.

Conflict of interest statement

None declared.

References

1. Cochlovius B, Braunagel M, Welschof M. Therapeutic antibodies. *Mod Drug Dis* 2003, **6**, 33–38.
2. Cai X, Garen A. 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* 1995, **92**, 6537–6541.
3. Kupsch JM, Tidman NH, Kang NV, Truman H, Hamilton S, Patel N, *et al*. Isolation of human tumor-specific antibodies by selection of an antibody phage library on melanoma cells. *Clin Cancer Res* 1999, **5**, 925–931., *Proc Natl Acad Sci USA*, **94**, 9261.
4. Noronha EJ, Wang X, Desai SA, Kageshita T, Ferrone S. Limited diversity of human scFv fragments isolated by panning a synthetic phage-display scFv library with cultured human melanoma cells. *J Immunol* 1998, **161**, 2968–2976.
5. Pereira S, van Belle P, Elder D, Maruyama H, Jacob L, Sivandham M, *et al*. Combinatorial antibodies against human malignant melanoma. *Hybridoma* 1997, **16**, 11–16.
6. Ridgway JB, Ng E, Kern JA, Lee J, Brush J, Goddard A, *et al*. Identification of a human anti-CD55 single-chain Fv by subtractive panning of a phage library using tumor and nontumor cell lines. *Cancer Res* 1999, **59**, 2718–2723.
7. Topping KP, Hough VC, Monson JR, Greenman J. Isolation of human colorectal tumour reactive antibodies using phage display technology. *Int J Oncol* 2000, **16**, 187–195.
8. Roovers RC, van der Linden E, de Bruine AP, Arends JW, Hoogenboom HR. Identification of colon tumour-associated antigens by phage antibody selections on primary colorectal carcinoma. *Eur J Cancer* 2001, **37**, 542–549.
9. De Kruif J, Terstappen L, Boel E, Logtenberg T. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. *Proc Natl Acad Sci USA* 1995, **92**, 3938–3942.
10. Kramer RA, Cox F, van der Horst M, van den Oudenrijn S, Res J, Bia J, *et al*. A novel helper phage that improves phage display slection efficiency by preventing the amplification of phages without recombinant protein. *Nucleic Acid Res* 2003, **31**, 11–59.
11. de Kruif J, Boel E, Logtenberg T. Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J Mol Biol* 1995, **248**, 97–105.
12. Boel E, Verlaan S, Poppelier MJ, Westerdaal NA, Van Strijp JA, Logtenberg T. Functional human monoclonal antibodies of all isotypes constructed from phage display library-derived single-chain Fv antibody fragments. *J Immunol Methods* 2000, **26**, 153–166.
13. Post TW, Liszewski MK, Adams EM, Tedja I, Miller EA, Atkinson JP. Membrane cofactor protein of the complement system: alternative splicing of serine/threonine/proline-rich exons and cytoplasmic tails produces isoforms that correlate with protein phenotype. *J Exp Med* 1991, **174**, 93–102.
14. van Kempen LCLT, Kersten-Niessen MJF, Bloemers HPJ, Swart GWM. Proteolytic cleavage of ALCAM: a gateway to melanoma metastasis. In: Abstract poster presented at the conference on Membrane-bound proteolytic enzymes and cancer, Palermo, Italy, 19–21 May 2001.
15. Ziegler-Heitbrock HW. Heterogeneity of human blood monocytes: the CD14+ CD16+ subpopulation. *Immunol Today* 1996, **17**, 424–428.
16. Gao C, Mao S, Ronca F, Zhuang S, Quaranta V, Wirsching P, *et al*. De novo identification of tumor-specific internalizing human antibody-receptor pairs by phage-display methods. *J Immunol Methods* 2003, **274**, 185–197.
17. Barratt-Boyes SM. Making the most of mucin: a novel target for tumor immunotherapy. *Cancer Immunol Immunother* 1996, **43**, 142–151.
18. Zhang X, Arvan P. Cell type-dependent differences in thyroid peroxidase cell surface expression. *J Biol Chem* 2000, **275**, 31946–31953.
19. Bruhl H, Cohen CD, Linder S, Kretzler M, Schlondorff D, Mack. Post-translational and cell type-specific regulation of CXCR4 expression by cytokines. *Eur J Immunol* 2003, **33**, 3028–3037.
20. Degen WG, van Kempen LC, Gijzen EG, van Groningen JJ, van Kooyk Y, Bloemers HP, *et al*. MEMD, a new cell adhesion molecule in metastasizing human melanoma cell lines, is identical to ALCAM (activated leukocyte cell adhesion molecule). *Am J Pathol* 1998, **152**, 805–813.
21. Uchida N, Yang Z, Combs J, Pourquie O, Nguyen M, Ramanathan R, *et al*. The characterization, molecular cloning, and expression of a novel hematopoietic cell antigen from CD34+ human bone marrow cells. *Blood* 1997, **89**, 2706–2716.
22. Konishi N, Tsujikawa K, Yamamoto H, Ishida E, Nakamura M, Shimada K, *et al*. Overexpression of leucocyte common antigen (LAR) P-subunit in thyroid carcinomas. *Br J Cancer* 2003, **88**, 1223–1228.
23. Yang T, Zhang JS, Massa SM, Han X, Longo FM. Leukocyte common antigen-related tyrosine phosphatase receptor: increased expression and neuronal-type splicing in breast cancer cells and tissue. *Mol Carcinogen* 1999, **25**, 139–149.