

2 Materials and Methods

2.1 Phage Library Screening

A cDNA expression library was screened according to the manufacturer's instructions (picoBlue Immuno Screening Kit, Stratagene). In brief, Epicurean *E. coli* XL1 Blue MRF' (XL1 Blue) bacteria were incubated with an appropriately diluted phage solution. This solution was mixed with top agar and poured onto NZY plates. The plates were incubated at 42°C for circa 3.5 hours until a dense bacterial lawn could be seen. Nitrocellulose membranes (Protran and Optitran membranes, Schleicher & Schuell) were wetted in isopropyl- β -D-thiogalactopyranoside (IPTG 10 mM in double-distilled water [ddH₂O], Invitrogen) solution. The membranes were placed onto the plates which are incubated at 37°C for another 3.5 hours.

The membranes' orientation was marked before they were removed and washed in Tween tris buffered saline (TTBS: 200 mM Tris, 110 mM NaCl, 0.05% Tween 20). Subsequently the membranes were blocked in 5% non-fat dry milk (NFDM) in phosphate buffered saline (PBS) overnight at 4°C. The plates are stored at 4°C.

The membranes were washed in TTBS and incubated with the patient's plasma which has been preabsorbed against *E. coli* lysate and was diluted in TTBS (overnight at 4°C). The membranes were washed several times before they are incubated with an alkaline phosphatase IgG Fc γ -specific secondary antibody (Jackson ImmunoResearch, diluted 1:1,000 in TTBS). Finally the membranes were washed and incubated in 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride (NBT, BCIP from Promega) colour development solution (developing buffer: 100 mM Tris·Cl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5).

The membranes were scored and positive phage plaques are cored out and stored in SM buffer (100 mM NaCl₂, 10 mM MgSO₄, 50 mM Tris·Cl, pH 7.5)

at 4°C. The selected phages were screened again until a single positive plaque could be cored out.

2.2 Plasmid Excision

The cDNA insert carrying plasmid was excised according to the manufacturer's instructions (ZAP-cDNA Gigapack Gold Cloning Kit II, Stratagene). Briefly, XL1 Blue were incubated with phage stock and ExAssist helper phage at 37°C. The solution is then heated to 65-70°C for 20 minutes and centrifuged. Finally SOLR cells were transformed with the excised plasmid and incubated on ampicillin (Sigma) LB bacterial plates.

2.3 Total RNA Isolation from Cultured Cells or Tissues

Cultured cells or small bits of tissue were homogenised with 4 M guanidine thiocyanate and phenol containing Trizol (Invitrogen) according to the manufacturer's instructions. In brief, after adding Trizol an appropriate amount of chloroform was added and the sample was mixed. After centrifugation the upper aqueous containing total RNA was recovered. The total RNA was precipitated by adding 2-propanol and centrifugation. Subsequently, the pellet was washed with 75% ethanol and briefly dried. Finally, the RNA was dissolved in RNase free ddH₂O and stored at -80°C.

2.4 Reverse Transcriptase Reaction

Reverse Transcriptase (RT, Superscript II, Invitrogen) was used for the first strand cDNA synthesis according to the manufacturer's instructions. In brief, a mixture of 1-5 µg total RNA and oligo dT (Roche Molecular Diagnostics) were

denatured at 70°C. The contents was chilled on ice and RT reaction buffer (250 mM Tris·Cl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, Invitrogen) , dithiothreitol (DTT, Invitrogen) and 10 mM deoxy nucleotide triphosphate mix (dNTP, Roche Molecular Diagnostics) are added. The tube is warmed to 42°C and the RT was added. After an incubation of 50 minutes, the enzyme was deactivated by heating to 70°C. RNA complementary to the cDNA was removed by adding RNase H for 20 minutes at 70°C.

2.5 Northern Blot

10 µg total RNA was mixed with the appropriate volume of RNA sample loading buffer with ethidium bromide (R4268, Sigma) and incubated at 65°C for 10 minutes. The samples and a size marker (Millenium Marker, Ambion) were loaded on an agarose methanal gel (2 g agarose, 19 ml 10x MOPS running buffer, 30 ml methanal) and electrophoresed in 1x MOPS running buffer(10x MOPS running buffer: 0.4 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA). Subsequently, the RNA was visualised under an UV light and a picture was taken. Finally, the gel was rinsed in RNase free water for 5 minutes. (Transfer see below.)

2.6 Southern Blot

The DNA was loaded into the wells of a 1% agarose tris-acetate EDTA (TAE) gel with ethidium bromide and separated by electrophoresis (50x TAE: 2 M Tris-acetate, 0.05 M EDTA, pH 8.3). After this, the gel was photographed and rinsed in ddH₂O for 5 minutes. Subsequently, the gel was incubated in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 30 minutes, washed with ddH₂O for 5 minutes, placed into neutralisation buffer (1.5 M NaCl, 0.5 M Tris·Cl, pH 7.0) for 30 minutes, and washed with ddH₂O for 5 minutes. (Transfer see below.)

2.7 Nucleic Acid Transfer

The gel was placed on top of sponges soaked in 10x SSC buffer (20x SSC buffer: 3.0 M NaCl, 0.3 M sodium citrate). A prewetted positively charged nylon membrane (Hybond-XL, Amersham Biosciences) was carefully placed onto the gel, followed by several layers of gel blot paper (GB002, Schleicher und Schuell) and a stack of paper towels. A weight was placed on top of this structure. The transfer was done overnight.

After disassembling the stack the membrane was removed and rinsed in 2x SSC for five minutes. The DNA or RNA was covalently bound to the membrane by UV-crosslinking (UV Stratalinker 2400, Stratagene). The membrane was stored at 4°C (DNA) or -80°C (RNA) until the hybridisation.

2.8 Hybridisation

The membrane was incubated in the appropriate amount of hybridisation solution (ExpressHyb, Clontech) at 60°C (Southern blot) or 68°C (Northern blot) in an hybridisation oven (Hybridiser HB-1D, Techne) for one hour. A 5 ml aliquot of the hybridisation solution was also placed in the incubator.

For the probe 25 ng DNA was labelled with 1.85×10^6 Bq ^{32}P alpha dCTP (NEN/Perkin Elmer Life Sciences) according to the manufacturer's instructions (Prime It II, Stratagene). The non-incorporated radioactive dCTP was removed with a sepharose column (Probe Quant G-50 micro column, Amersham Biosciences). If the incorporated amount was above 25% of the total radioactivity, the probe was boiled for 10 minutes, chilled on ice for 30 seconds and then injected into the pre-heated 5 ml aliquot of hybridisation solution. The 5 ml were added to the tube with the membrane. Subsequently, the membrane was incubated with the probe from one hour to overnight.

The radioactive hybridisation solution was discarded and the membrane was incubated with 2x SSC buffer with 0.1% SDS at room temperature for 10

minutes. This step was repeated once. For the final washing step the oven was set at 50°C and the membrane was incubated with 0.1x SSC buffer with 0.1% SDS for 30 minutes. If the background of the membrane appeared to be high, additional washing steps were carried out. Finally, the membrane was wrapped into food service film and exposed to film (X-Omat AR, Kodak) at -80°C.

2.9 Colony Hybridisation Screening

A phage expression library was plated as described above (see phage library screening protocol) and incubated at 42°C until the plaques had reached the desired size. Nylon membranes (Nytran supercharged, Schleicher & Schuell) were put onto the plates for 30 seconds. The membranes were incubated in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 5 minutes and then washed in neutralisation buffer (1.5 M NaCl, 0.5 M Tris·Cl, pH 7.0). A duplicate membrane was made, it remained 3 minutes on the plate but was otherwise treated exactly the same.

For the hybridisation the membranes were put into a heat sealable bag with prehybridisation solution (Molecular Research Center) into a water bath at 65°C. After an incubation of at least 30 minutes the prehybridisation solution was replaced by the hybridisation solution (MRC) with the labelled DNA probe (DNA labelling see hybridisation protocol). The sealed bag was placed into the water bath at 65°C for at least 1 hour.

The membranes were removed from the bag and washed in 2x SSC at room temperature until the radioactive background reaches the desired level. The membranes were wrapped into food service film and exposed to film (X-Omat AR, Kodak) at -80°C.

Phage plaques that appeared positive in duplicate were cored, further purified, the phagemid was excised (see plasmid excision) and eventually the insert was sequenced.

2.10 Recombinant Glutathione S-Transferase Fusion Protein

The glutathione S-transferase (GST) fusion protein was produced with a pGEX vector according to the manufacturer's instructions (Amersham Biosciences). In brief, the gene of interest was cloned into the multiple cloning site of the vector. One transformed bacteria colony was picked and grown overnight in LB media with ampicillin at 37°C. The bacteria were diluted and grown to an optical density of 1 at 600 nm. Protein production was induced for several hours by adding IPTG to a final concentration of 0.1 mM. Subsequently the cultures were pelleted by centrifugation.

The bacterial pellets were frozen and stored at -20°C. After thawing the bacteria were resuspended in cold PBS and sonicated. Triton X-100 is added to aid solubilisation of the protein. The bacterial debris was separated by centrifugation and the supernatant was incubated with GST sepharose beads (Amersham Biosciences). After several washes the fusion protein was eluted from the beads with glutathione buffer (10 mM reduced glutathione in 50 mM Tris·Cl, pH 8.0). A protein assay (BioRad) was carried out to determine the protein. To verify the integrity of the protein a sample was run on a minigel which was stained with Coomassie blue solution (50% methanol, 40% ddH₂O, 10% ethanoic acid, 0.05% [w/v] Coomassie brilliant blue).

2.11 Enzyme-Linked Immuno Sorbent Assay (ELISA)

Glutathione S-transferase (GST)-ML-IAP and GST full-length recombinant proteins were produced with the pGEX 5X-3 vector (Amersham Biosciences) according to the manufacturer's procedures. ELISA plates (Corning) were coated overnight at 4°C with 500 ng of GST-ML-IAP or GST protein in a carbonate buffer (15 mM Na₂CO₃, 30 mM NaHCO₃, 0.02% (w/v) NaN₃, pH=9.6). The wells were blocked overnight at 4°C with 2% NFDM/PBS, washed, and incubated in duplicate with 50 µl of patient sera diluted 1:100-1:10,000 in 2% NFDM/PBS for 2

hours at room temperature. A polyclonal goat anti-human pan IgG (Jackson ImmunoResearch, 1:1,000 dilution), monoclonal mouse anti-IgG1 (Zymed, 1:500 dilution), or monoclonal mouse anti-IgG4 (Southern Biotechnology Associates, 1:500 dilution) conjugated to alkaline phosphatase in 2% NFDM/PBS was added at room temperature for 1 hour, and the plate was developed with p-nitrophenyl phosphate (pNPP, Sigma). The values reported were the mean absorbance at 405 nm of GST-ML-IAP measured 25 minutes after adding the substrate on an ELISA reader (Spectra Max 190, Molecular Devices). All timepoints showed no reactivity against GST.

As an internal control allergenic extract from *Candida* was used (Hollister-Stier). The ELISA was carried out as described above with a 1:10 dilution of the extract for coating the ELISA plate and a plasma sample dilution of 1:1,000 in 2% NFDM/PBS.

2.12 T Cell Assays

Necrotic metastases were processed to single cell suspension by mechanical digestion and placed into short-term culture (RPMI 1640 with 10% foetal calf serum). Non-adherent cells were harvested after several hours and cryopreserved for immune analysis. Approximately 25% of these suspensions were CD3⁺ T lymphocytes, with equal proportions of CD4⁺ and CD8⁺ cells. The melanoma cell line M34 was established from the adherent cells of the heavily infiltrated calf metastasis.

Proliferative responses to ML-IAP were evaluated by first sorting CD8⁻ cells from the processed calf metastasis and expanding these cells in media supplemented with 10 U/ml IL-2 for four weeks to obtain a 95% pure population of CD4⁺ T cells. Five replicates of 1×10^5 cells were then incubated with GST-ML-IAP or GST (1 µg/ml) for four days and tritium-labelled thymidine uptake measured. CD4⁺ T cells from a healthy donor served as a control.

MHC class I-restricted responses were evaluated by first identifying candidate HLA-A*0201 (one of the alleles expressed by K030) -binding peptides using standard algorithms. Two peptides (Sigma-Genosys), JS34 (SLGSPVLGL) and JS90 (RLASFYDWPL), showed high affinity binding in T2 cell-based assays.

Soluble HLA-A2 tetramers were prepared as described with 2-microglobulin and JS34, JS90, or L11 (LLFGYPVYV), a peptide derived from HTLV-1 tax, and conjugated to phycoerythrin. Cells harvested from metastases were stained with tetramers and FITC-conjugated anti-CD8 mAb (Dako) for 30 minutes at room temperature and analysed on an EPICS XL flow cytometer (Beckman Coulter).

For ELISPOT analysis, ImmunoSpotplates (Cellular Technology Limited) were coated overnight at 37°C with 10 µg/ml of anti-IFN-γ mAb (Mabtech). Cells harvested from metastases were mixed 1:1 with peripheral blood mononuclear cells (as a source of antigen presenting cells) and plated at 2×10^5 cells/well with 1 µg/ml of JS34, JS90, L11 tax, or HIV-1 reverse transcriptase-polymerase I476 (ILKEPVHGV) peptide. After 24 hours at 37°C, the wells were washed and then incubated with 1 µg/ml biotin-conjugated anti-IFN-γ mAb (Mabtech) followed by streptavidin-alkaline phosphatase (Mabtech). Spots were developed using BCIP/NBT (Promega) as a colour development substrate and counted with an ImmunoSpot microplate reader (Cellular Technology Limited).

For CTL assays non-adherent cells harvested from metastases were cultured with irradiated M34-ML-IAP cells in media plus 10 U/ml IL-2 for three weeks and then evaluated in a four-hour ^{51}Cr release assay using M34 and M34-ML-IAP cells as targets. Percent specific lysis was calculated using the formula:
$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}$$

2.13 Construction of a Retroviral Vector and Production of VSV-G-Pseudotyped Retroviral Particles

The gene of interest was cloned into the pUC19/MMLV-based, replication-deficient retroviral vector MFG.S between the NcoI/XbaI and BamHI sites so that the position of the initiator ATG was maintained.

The production of the amphotropic retroviral particles was carried out as described by Ory et al. [166] with 293 GPG cells that express MMLV gag.pol constitutively and VSV-G under a tetracycline-repressed promoter. In brief, an appropriate number of 293 GPG packaging cells was plated with tetracycline containing medium. The next day the cells were washed with serum-free medium (Optimem, Invitrogen) and incubated with a suspension of the plasmid, Lipofectamine (Invitrogen) and Optimem. Circa 8 hours posttransfection regular DMEM (10% foetal calf serum) is added; after 24 hours this mixture was replaced with regular DMEM.

The viral supernatant was harvested and filtered through a 0.45 µm low protein binding membrane (Pall Gelman) and stored at -80°C. The medium was replaced with regular DMEM. This step was repeated during the consecutive days until the majority of the 293 GPG cells is dead (This was normally the case after not more than 5 days.).

The viral supernatants were thawed and concentrated by ultracentrifugation at 50,000 g and 4°C for 1.5 hours. The supernatant is discarded and the viral pellet is resuspended in a small volume of medium or 10% Hank's balanced saline solution (HBSS) in PBS. The tubes were incubated overnight at 4°C. On the next day the viral solution was aliquoted and stored at -80°C until usage.

The retroviral infection of the target cells was typically carried out for 4-6 hours in the appropriate medium in the presence of 8 µg/ml hexadimethrine bromide (Sigma).

2.14 Anti-ML-IAP Monoclonal Antibody and Immunohistochemistry

Mice were immunised with formalin-treated, dialysed GST-ML-IAP, and hybridomas were generated with standard techniques. A clone specific for ML-IAP (3F9, IgG1) and suitable for use in tissue sections and immunoblots was identified.

Hybridoma cells were cultured in a special two-chambered flask (Integra CL 1000, Integra Biosciences) and the antibody-containing supernatant was collected, centrifugated and frozen. For purification a protein G-based antibody affinity column was used according to the manufacturer's instructions (MAbTrap, Amersham Biosciences). The antibody concentration was determined by ELISA. In brief, an ELISA plate was coated with polyclonal hamster anti-mouse IgG (Jackson ImmunoResearch) and incubated with the purified monoclonal antibody (mAb) or a murine myeloma derived IgG1 (Calbiochem) that served as a standard. A polyclonal hamster anti-mouse IgG labelled with horseradish peroxidase (HRP)(Jackson ImmunoResearch) was used for detection. (The immunisation and hybridoma fusion was carried out by Edward A. Greenfield in the DFCI's Hybridoma Core Facility. The author provided the recombinant protein, screened and tested the supernatants, cultured the hybridoma cells, purified the mAb and performed quality controls.)

For immunohistochemistry, 5 µm thick formalin-fixed, paraffin-embedded tissue sections were deparaffinised, pre-treated with 10 mM citrate, pH 6.0 (Zymed) in a steam pressure cooker (Decloaking Chamber, BioCare Medical) per the manufacturer, and washed in distilled water. Next, slides were pre-treated with Peroxidase Block (Dako) for 5 minutes at room temperature in a hydrated chamber to quench endogenous peroxidase activity and blocked with a 1:5 dilution of goat serum in 50 mM Tris, pH 7.4 for 20 minutes. The slides were then incubated with a 1:50 dilution of 3F9 in 50 mM Tris, pH 7.4 and 3% goat serum for 1 hour, washed, and incubated with a goat anti-mouse Ig HRP-conjugated antibody (Envision Detection Kit, Dako) for 30 minutes. The slides were developed with a diaminobenzidine chromogen kit (Dako) per the manufacturer,

placed in an enhancing solution (Zymed) for five minutes, and counterstained with hematoxylin. In some experiments, mAbs to CD4, CD8, or CD20 were used.

2.15 Whole Cell Lysates

Whole cell lysates were prepared by washing cells with PBS and incubating them in a lysis buffer that contains the detergent NP-40 and protease inhibitors (PBS with 0.5% (v/v) NP-40/IGEPAL CA-630, 1 µg/ml pepstatin, 10 µg/ml leupeptin, 174 µg/ml PMSF, 100 µg/ml soybean trypsin inhibitor, 65.5 µg/ml aminocaproic acid. All reagents from Sigma.). After a 30 minute incubation on ice with agitation the samples are centrifugated and the supernatant is collected and stored at -20°C. The concentration of protein is determined with a BioRad protein assay.

2.16 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

Usually 10% or 12% resolving gels with a 3.9% stacking gel were prepared (4x Tris:Cl/SDS resolving buffer: 1.5 M Tris:Cl, 0.4% SDS; 4x Tris:Cl/SDS stacking buffer, pH 6.8: 0.5 M Tris:Cl, 0.4% SDS; 30% acrylamide/0.8% bisacrylamide; 5x electrophoresis buffer: 0.125 M Tris base, 0.96 M glycine, 0.5% SDS).

An appropriate amount of protein was diluted with PBS and 6x denaturing buffer (70% [v/v] 4x Tris:Cl/SDS, pH 6.8, 30% [v/v] glycerol, 10% [w/v] SDS, 0.6 M DTT, 0.012% bromphenol blue). All samples were boiled for 5 minutes and then electrophoresed on a denaturing polyacrylamide gel. A stained protein ladder was used for determining the weight of protein bands (Invitrogen).

2.17 Immunoblotting (Western)

Protein from the gel was transferred to a PVDF membrane (Millipore) with a wet transfer system (BioRad) according to the manufacturer's instructions (transfer buffer: 25 mM Tris, pH 8.3, 192 mM glycine, 20% [v/v] methanol). The membrane was blocked overnight at 4°C in 5% NFDM/PBS. The appropriate antibody was solubilised in 5% NFDM/TTBS and incubated at room temperature for 1 hour. After washing with TTBS the secondary HRP-labelled antibody in 5% NFDM/TTBS was applied for 1 hour. Several washes with TTBS were followed by adding the substrate (Western Lightning kit NEN/Perkin Elmer) and by exposure to film (X-Omat Blue, Kodak).

If necessary, blots were stripped by incubating them with blot erasure buffer (62.5 mM Tris-Cl, pH 6.8, 2% [w/v] SDS, 100 mM 2-mercaptoethanol) at 65°C in sealable plastic bags submerged in a water bath.