2. Materials and Methods

2.1 Chemicals

If not indicated otherwise all chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany)

2.2 Cloning and expression of recombinant proteins used for immunisations

2.2.1 FeLV-A p15E ectodomain

DNA from FeLV-A producing FEA feline embryonic fibroblast cells was isolated using a Qiagen DNA isolation kit. Using the forward primer FeLV-A p15E forw. and the reverse primer FeLV-A p15E rev., a sequence corresponding to the ectodomain of the transmembrane envelope protein p15E (amino acids 476-583) was amplified by polymerase chain reaction and cloned into the pCal-n vector (Stratagene, Europe, Amsterdam, Netherlands). *E. coli* BL21 DE3 cells were transformed and p15E N-terminally fused to a 4 kDa calmodulin binding protein (CBP) was produced. The fusion protein was purified by calmodulin resin affinity chromatography (Stratagene). Protein to be used for immunisation and for inhibition experiments was extensively dialysed against phosphate-buffered saline (PBS).

2.2.2 ΔISU p15E and p15E/gp41 I and II hybrid proteins

The p15E backbone was amplified from the pCaln-p15E vector (aa476-512 and aa530-539 and 558-583). The DNA sequence of the immunosuppressive domain (Cianciolo et al., 1985; Denner 1998) and the peptide J and K (Nick et al., 1990) were deleted by multi step PCR. Using primers FeLV-A forward and primer p4 fragment 1 corresponding to aa476-512 and 530-539 was amplified containing an N-terminal *Bam* HI restriction site and a C-terminal *Not* I restriction site. The fragment 1 obtained from that PCR is deleted for the DNA sequence of isu (aa513-529) and bears the DNA sequence of the cystein loop (aa531-539). Using primers p5 and FeLV-A reverse fragment 2 corresponding to aa558-583 was amplified lacking the C-terminal opposing sequence of isu (aa540-558). The fragment 2 contained an N-terminal *Not* I restriction site and a C-terminal *Eco* RI restriction site.

The p15E/gp41 I and II hybrid proteins consist of fragment 1 obtained for the generation of FeLV-A ΔISU p15E (2.2.1) and the DNA sequence of the E2 region of HIV-1 IIIB gp41 amplified from the pNL4-3 vector (aa645-aa680 for p15E/gp41 I and aa636-aa680 for p15E/gp41 II). For the amplification of the gp41 E2 sequences the gp41 reverse primer and p5 hybrid I or p5 hybrid II primers were used generating an N-terminal *Not* I restriction site and a C-terminal *Eco* RI restriction site.

The amplified sequences for FeLV-A ΔISU p15E and for the p15E/gp41 hybrid proteins I and II were ligated using inserted restriction sites *Not*I, *Bam*HI and *Eco* RI and cloned into the pCal-n vector

(Stratagene, Europe, Amsterdam, Netherlands). *E. coli* BL21DE3 cells were transformed and the hybrid protein fused to the 4 kDa calmodulin binding protein (CBP) was produced.
Due to the insolubility of recombinant proteins FeLV-A ΔISU p15E, p15E/gp41 hybrid protein I and II calmodulin resin affinity chromatography was inefficient. Thus the resulting *E.coli* pellet, containing the hydrophobic target protein, was washed intensively, air dried, pulverised and suspended in PBS.

2.3 Experimental animals

2.3.1 Immunisation of rats and goat with the FeLV-A p15E ectodomain

Wistar rats were obtained from a commercial supplier and housed in groups under barrier conditions. To generate p15E-specific antibodies, 12 rats and one goat were immunised intramuscularly (i.m.) and subcutaneously (s.c.) three times (at 0, 3 and 6 weeks unless stated otherwise) with 0.5 mg of the affinity-purified recombinant fusion protein in 0.4 ml buffered saline emulsified in 0.4 ml incomplete Freund's adjuvant.

2.3.2 Immunisation of rats with ΔISU p15E and p15E/gp41 I and II hybrid proteins

Wistar rats were obtained from a commercial supplier and housed in groups under barrier conditions. Rats were immunised intramuscularly (i.m.) twice (at 0 and 3 weeks unless stated otherwise) with 0.1 mg recombinant protein in 0.5ml buffered saline emulsified in 1.2 ml Montanide® ISA 720 (Seppic, France, lot number 143521). Three rats were immunised with FeLV-A ΔISU p15E (group70), 10 rats were immunised with p15E/gp41 hybrid protein I (groups 71, 79 and 91) and 4 rats were immunised with hybrid protein II (group 90). Additionally 3 rats were immunised with p15E/gp41 I hybrid protein as described and boostered once with 0.1mg gp41-derived peptide (aa656-680) emulsified in 0.25ml Montanide® ISA 720.

2.3.3 Immunisation of rats with p15E and p45

Two immunisation experiments were performed using Wistar rats. In the first experiment, 9 rats were immunised twice intramuscularly (i.m.) and subcutaneously (s.c.) (at weeks 0 and 3). For immunisation recombinant p15E of FeLV-A was prepared as described. In the first experiment 1 dose Leucogen, containing 0.1mg p45 plus Quil-A and aluminium hydroxide as adjuvant (Virbac, lot number 80986902143521) was given alone or mixed with 0.5mg p15E. Freund´s adjuvant was used when p15E was injected alone. In the second experiment 18 rats were immunised i.m. and s.c. with 0.1mg or 0.5mg p15E alone, with one dose Leucogen p45 alone or with a mixed preparation of 0.1mg

or 0.5mg p15E and one dose Leucogen p45 at week 0 and 3. Immunisation with p15E alone was performed at a dilution 7:3 in Montanide® ISA 720 (Seppic, France, lot number 143521).

2.3.4 FeLV-A infected pet cats, immunisation of cats with p15E and p45

Sera from infected cats were obtained from household cats in Germany at the time of first diagnosis of infection using a commercial p27 Gag antigen detection assay (Feline leukemia virus antigen test kit, Symbiotics, USA).

6-10 month old cats, obtained from the University of Düsseldorf and housed in groups of 3, were immunised intramuscularly (i.m.) twice (at weeks 0 and 3). For p15E immunisations Montanide® ISA 720 (Seppic, France, lot number 143521) was used as adjuvant mixed with p15E at a ratio of 3:7. 6 cats were immunised with 0.5mg p15E, 3 cats were immunised with one dose Leucogen® p45 each (Virbac, lot number 80986902143521) and 2 cats were immunised with a mixed preparation of 0.5mg p15E and one dose Leucogen® p45. A group of 3 cats did not receive any immunisation serving as positive control group. The cats were challenged oronasal with 4 doses of $1x10^6$ ffu/ml FeLV-A Glasgow strain (kindly provided by Dr, O. Jarrett, Department of Veterinary Pathology, University of Glasgow Glasgow, UK) at four consecutively days. Blood was taken 3 days before challenge and 10, 30, 60, 80 and 100 days after challenge.

2.4 Purification of antisera by p15E affinity and proteinG columns

Purified p15E coupled to CNBr-activated Sepharose (Pharmacia) was used to adsorb specific antibodies from serum that were then eluted using Tris-glycine buffer, pH 2.5 and dialysed against PBS. Protein G columns (Montage®, Millipore) were used in parallel to purify IgG as described by the manufacturer.

2.5 Characterisation of antisera in Western blot and ELISA

SDS-PAGE and Western blotting were performed as described previously (Tacke et al., 2001) using 1µg-20µg of purified recombinant protein per lane. Pre- and post-immunisation experimental animal sera were titrated in ELISA using as antigen affinity-purified recombinant p15E protein, FeLV-A ΔISU p15E protein, Leucogen p45, p15E/gp41 I hybrid protein or HIV-1 gp41 E2 peptide (aa656-680). Plates (Nunc) were coated for 1h at 37°C or in case of Leucogen® p45, p15E/gp41 I hybrid protein and HIV-1 gp41 E2 peptide at 37°C for 18 hours with protein diluted in PBS (100ng/well). ELISA plates then were washed once with PBS containing 0.1% Tween 20 and blocked for 1 h at room temperature with PBS containing 0.1% Tween 20 and 5% BSA. Serum samples, diluted in PBS containing 2.5% BSA and 0.1% Tween-20, were added to the ELISA plate at an initial dilution of

1:100 or 1:1000 and diluted further in three- or four-fold steps. After incubation for 1h at 37°C, ELISA plates were washed three times in PBS containing 0.1% Tween 20 and a horseradish peroxidase conjugated secondary antibody specific for human IgG (Sigma-Aldrich), goat IgG (Dako), rat IgG (Dako) or cat IgG (Bethyl, USA), diluted 1:2000-1:8000 in PBS containing 2.5% BSA and 0.1% Tween-20, was added. Incubation for 1h at 37°C was followed by five washes with PBS containing 0.1% Tween 20. Finally, ELISA plates were developed by addition of OPD (*a*-phenylenediamine dihydrochloride) diluted in PBS (50µg/well) plus 0.1 % H_2O_2 and stopped after 10 minutes by addition of 30µl H_2SO_4 (5N). Antibody endpoint titres are reported as the dilution giving an $OD_{492/620nm}$ reading above the background of pre-immune sera. Protein-specific antibody endpoint titres are reported as the dilution giving an $O.D._{492 / 620nm}$ reading above that of preimmune sera.

2.6 Epitope mapping on FeLV-A p15E and HIV-1 gp41

Peptides corresponding to the entire p15E of FeLV-A, Glasgow strain or to the entire gp41 of HIV-1IIIB were synthesized as 15-mer peptides overlapping by 13 amino acids and were covalently linked to a cellulose sheet (Jerini Biotools). Sera diluted 1:500-1:1000 were incubated with the membrane for 2h, washed three times for 15 min with Tris-buffered saline, pH 7.5 containing 0.05% Tween 20 (Sigma) and incubated for 2 h with a peroxidase-conjugated secondary antibody diluted 1:3500-1:10,000. Binding was detected using a chemiluminescence detection solution (ECL, Amersham Pharmacia Biotech).

2.7 Immunofluorescence on FEA cells

FeLV-A producing FEA cells were grown on chamber slides, washed three times with PBS and fixed with 3.5% formaldehyde. Unspecific binding sites were blocked with 5% BSA in PBS for 20 minutes followed by washing with PBS. Cat sera were applied in 2.5% BSA/PBS at a dilution of 1:1000 and incubated at 37°C for 1 h. After five washes with PBS the cells were incubated with FITC-labeled goat anti-cat IgG (Bethyl, USA). Finally, the cells were embedded in Prolong® antifade reagent (Molecular Probes) and the surface fluorescence was analysed by confocal microscopy (Zeiss, LSM510). Unspecific cell fluorescence at 543nm was subtracted from the specific signal at 488nm.

2.8 Retroviral neutralisation assays

2.8.1 FeLV-A neutralisation assay with FEA cells

The virus stock for the neutralisation assay was prepared as cell-free supernatant from FEA cells infected with the FeLV-A Glasgow strain (kindly provided by M. Reinacher, Giessen, Germany and O. Jarrett, Glasgow, UK). The stock was titrated on uninfected FEA cells and shown to have a titre of $10^{4.76}$ $TCID_{50}$/ml. Neutralisation assays were performed as follows. FEA cells were seeded at 6000 cells per well into 96-well microtitre plates one day before use. Preimmune and immune sera were heat-inactivated at 56°C for 30 min. 50µl of virus was added to serial dilutions of serum or purified immunoglobulin and incubated for 30 min at 37°C before transfer to the cells. Alternatively, the dilution of serum was held constant at 1:5 and the virus was serially diluted. After 3 days incubation, cells were freeze-thawed three times and a lysis buffer containing 20 mg/ml of proteinase K in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 8.4) was added. The cells were incubated for 3h at 56°C followed by 10 min at 95°C to inhibit proteinase K activity. Provirus was then quantitated by PCR as described in 2.9.1.

2.8.2 HIV-1 and PERV virus neutralisation assays with C8166 cells

The virus stock for the neutralisation assay was prepared as cell-free supernatant from C8166 cells infected with HIV-1IIIB and titrated on uninfected C8166 cells. 50µl cell-free virus-containing supernatant ($1x10^3$ $TCID_{50}$) were used to infect $5x10^4$ C8166 cells seeded into a well of a 96 U-well microtitre plate in a total volume of 100µl. Preimmune and immune sera were heat-inactivated at 56°C for 30 min. Serial dilutions of the sera were added to the virus and incubated for 45 min at 37°C before transfer to the cells. After 72 hours incubation (37°C, 5% CO_2), cells were freeze-thawed three times and a lysis buffer containing 20 mg/ml of proteinase K in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 8.4) was added. The cells were incubated for 3h at 56°C followed by 10 min at 95°C to inhibit proteinase K activity. Provirus integration in infected cells was measured by quantitative real-time PCR as described in 2.9.2. Neutralisation assays on macrophages with HIV-1 isolates Bal and SF162 were carried out on differentiated macrophages as described previously (Ruppach et al., 2000) and subsequently analysed as described above. For neutralisation assays performed with immunoglobulins, protein G columns (Montage®, Millipore) were used to purify IgG as described by the manufacturer.

For infection of C8166 cells with PERV 50µl cell-free virus-containing supernatant of PERV/5°-infected 293 cells ($1 \times 10^{4.31}$ $TCID_{50}$/ml) was added to the cells and identical procedures were carried out as described for HIV-1 neutralisation assay. Quantitative real-time PCR analysis for PERV provirus integration was performed as described in 2.9.3.

2.9 Determination of retroviral provirus integration by real time PCR

2.9.1 FeLV-A real time PCR

An internal probe FAM-5´-TTAAGCACCTGGGCCCCGGC-3´-DQ (Eurogentec) was used together with FeLV-specific primers. The sense primer 5´-TCAAGTATGTTCCCATGAGATACAA-3´ and antisense primer 5´-GAAGGTCGAACTCTGGTCAACT-3´ were used to amplify and to quantify a 185bp product from the exogenous U3 sequence in the LTR region of the FeLV-A provirus genome. The 25µl reaction mixture consisted of 1x PCR buffer with 1mM $MgCl_2$, 0.5µM each of dATP, dCTP, dGTP, dTTP, 5pmol of each primer, 5pmol of probe, 1.25 U Amplitaq Gold polymerase and 2µl lysis mixture obtained from the FeLV-A neutralisation assay (2.8.1). The thermal cycling conditions used were 12 minutes at 95°C followed by 50 cycles of 1 minute at 95°C, 1 minute at 59°C and 30 seconds at 72°C in a Stratagene MX4000 machine if not indicated otherwise.

2.9.2 HIV-1 real-time PCR

2µl of cell lysate from neutralisation assays were used as template in a gp41-specific real-time PCR using primers SK68i (5´-GGARCAGCIGGAAGCACIATGG-3´), SK69i (5´-CCCCAGACIGTGAGI TICAACA-3´) and the probe 6Fam-TGACGCTGACGGTACAGGCCAGAC-dabcyl (Taqman Universal Mastermix, Applied Biosystems). The assay was performed using a Stratagene MX4000 (55cycles, annealing at 55°C, no elongation phase) (Schweiger et al., 1997).

2.9.3 PERV real-time PCR

2µl of cell lysate from neutralisation assays were used as template in a PERV gag specific real-time PCR using primers PERV real s (5'-TCCAGGGCTCATAATTTGTC-3´), PERV real_as (5'-TGATGGCCATCCAACATCGA -3´) and the probe 6Fam- AGAAGGGACCTTGGCAGACTTTCT -dabcyl (Taqman Universal Mastermix, Applied Biosystems). The assay was performed as described for HIV-1 real-time PCR (2.9.2)

2.9.4 Calculation of provirus integration and neutralisation efficiency

In order to obtain the percentage of provirus integration, the Δct value of the control serum and the immune serum was calculated and used in the formula $(2^{-\Delta ct})$ x100%. The FeLV-A, PERV and HIV-1 neutralisation efficacy was calculated as percentage of provirus integration subtracted from 100%.

2.10 Quantification of p27 antigen and provirus load in FeLV-A infected cats

Sera obtained from household cats or from FeLV-A challenged cats were tested for productive infection using a commercial p27 Gag antigen detection assay (Feline leukemia virus antigen test kit, Symbiotics, USA). Provirus load was determined by DNA purification from 100µl peripheral blood, analysed for provirus integration in a FeLV-A real time PCR and calculated in copies/µl blood by the usage of a FeLV-A plasmid standard.

2.11 Generation of hybridoma cells from rat lymphocytes

Shortly after the spleen was excised from a rat producing the antibody of choice, it was teased in ice cold serum-free medium (D-MEM without supplements) and the resulting cell suspension was passed through a Falcon 70 micron cell filter and suspended in 50 ml of ice cold D-MEM without supplements. The cells were centrifuged and washed three times at 4° C. Afterwards cells were resuspended in 10 ml D-MEM without supplements and viable cells were counted. The cells were kept on ice. Concurrently with the spleenocytes, the mouse myeloma cells P3X63Ag8.653 (maintained at $<1x10^6$cells/ml) were centrifuged and washed three times. They were resuspended in D-MEM without supplements and viable cells were counted. An appropriate number of myeloma cells were added to the entire volume of spleen cells in a ratio of 1:5 and centrifuged together. All supernatant was aspirated and the resulting pellet was suspended by tapping the end of the tube. The tube was placed in a container of warm water (37°C) and 1 ml of 37° C 50% w/v PEG was gradually added over a period of 60 seconds, while tapping the side of the tube to achieve thorough mixing. Additional 60 seconds the mixing was continued subsequently followed by diluting the PEG/cell mixture slowly by adding dropwise 2 ml of D-MEM without supplements over a 2 minute time span. Adjacent 8 ml 37° C D-MEM supplemented with 10% FCS, HAT, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 U penicillin, 50 µg streptomycin and 50 µM 2-ME were added over a 4 minute period and finally the total volume was equilibrated to 50 ml using D-MEM with supplements (20%FCS). The cells were centrifuged at 4°C and resuspended in medium at the appropriate volume to bring the cells to a concentration of $1.5x10^6$cells/ml. From this suspension 150 µl were added to each well of 6x96 well plates (Köhler and Milstein 1975).The day of the fusion was considered day 0 and the fusion plates were examined at 24-48 hours for any abnormalities (i.e. bacterial contamination). On day 7, wells were inspected visually and then fed with 150 µl of D-MEM with supplements (20%FCS) on days 7, 11 and thereafter as needed. The cultures were examined visually at each feeding. Once a majority of wells appear 50% confluent for growth, supernatants were harvested for screening in ELISA and cells were fed at this time. 21 days after fusion, the HAT component in the medium was replaced by HT and cells were transferred to 24well plates. Corresponding cells to hybridoma cell culture supernatants tested positive in screening ELISA were singularized twice to obtain monoclonal antibodies. Finally HT selection medium was replaced by D-MEM with supplements containing 10% FCS and positive hybridoma cells were maintained in T75 flask.