2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell cultures

Human melanoma cell lines

The human cell lines A-375 (Giard *et al*, 1973) and Mel-HO (Holzmann *et al*, 1988), SK-Mel-13, SK-Mel-19, SK-Mel-23 (Carey *et al*, 1976), MeWo (Bean *et al*, 1975) and Mel-2a (Bruggen *et al*, 1981) were used for subsequent investigations.

A-375/mock and A-375/Bcl-2 as well as Mel-HO/mock and Mel-HO/Bcl-2 had been established by transfection of parent A-375 and Mel-HO with pIRES plasmid (Clontech, Palo Alto, Calif., USA) or with a pIRES construct enclosing a full length cDNA for mouse Bcl-2 (pIRES-Bcl-2), as described previously by Raisova et al. 2001. The tetracycline-regulatable melanoma cell line SKM-13-Tet-On derived from stable transfection of pTet-On plasmid (Clontech) into parent SK-Mel-13 cells described by Eberle et al. 2003. Stable SKM-13-Bcl-x_L and SKM-13-mock cell clones derive from transfection of SKM-13-Tet-On with pTRE-Bcl-x_L and pTRE-1 (Clontech), respectively, as described by Hossini *et al,* 2003. Expression of Bcl-x_L was induced in these cells by addition of 2 μg/ml doxycycline to the groth medium.

Selection of TRAIL-resistant melanoma cells

Parent A-375, SK-Mel-13 and Mel-HO melanoma cell lines were seeded in culture flasks (1x 10^6 cells/flask) and continuously treated with 100 ng/ml TRAIL for 8-10 weeks. Growth medium with new TRAIL was changed 2-times a week. When selected cells had reached confluence, they were passaged and continuously kept in growth medium with 5 ng/ml TRAIL (selected cells). For recovering TRAIL sensitivity, selected cultures were subjected to TRAIL withdrawal for 1 month (W1) or for 3 months (W3).

Normal human melanocyte cell cultures

Several cultures of normal human melanocytes (NHM) were established from different foreskins by trypsin digestion and were cultivated as described by Eberle at al. 1999 in serum-free melanocyte growth medium (Eberle *et al*, 1999).

2.1.2. Cell culture media and solutions

For growth and cultivation of melanoma cell lines following media and solutions used:

Dulbecco's modified Eagle' medium (DMEM / 10 % FCS).

1. DMEM (incl. 4 mM L-glutamine, 25 mM glucose) (Invitrogen) 500 ml

2. Fetal calf serum (FCS) (Seromed-Biochrom) 50 ml

3. 10,000 IE penicillin/ 10,000 µg/ ml streptomycin (Seromed-Biochrom) 5 ml)

The supplemented growth medium was stored for up to four weeks at 4 °C.

Trypsin solution

Trypsin (Seromed-Biochrom) 1.5 g

Phosphate buffered saline (PBS) (Seromed-Biochrom) 500 ml

The solution was stored in aliquots of 10 ml at -20 °C.

Freezing solution for melanoma cells

DMEM supplemented with 30% FCS and 10% Dimethylsulfoxide (DMSO)

2.1.3. Bacterial growth medium and antibiotics

LB-Medium (GibcoBRL) for agar plates

Trypton	10 g
Yeast Extrakt	5 g
NaCl	10 g
NaOH (1M)	1 ml
H_2O	up to1000 ml
Ampicillin	50 µg/ml
Agar	14 g

For liquid LB medium agar was omitted

2.1.4. Antibiotics (stock solutions)

Ampicillin (Gibco)	50 mg/ml diluted in sterile water
Geneticin (Gibco)	100 mg Geneticin in 2 ml PBS
Hygromycin (Boehringer)	50 mg/ml in sterile filtered water

Doxycyclinhydrochloride (Sigma)	1mg/ml diluted in sterile filtered water

2.1.5. Apoptosis stimulation agents

*Killer*TRAIL, (soluble, human, recombinant) Alexis

CH-11 (agonistic monoclonal Anti-Fas antibody) Immunotech

TNF-α (soluble, human, recombinant) Sigma

2.1.6. Enzymes

Enzymes used for restriction analysis (all from GibcoBRL)

Enzyme	Buffer	Temperature °C
Eco RV	3	37
Not I	3	37
Eco RI	3	37
Nco I	4	37
Xho I	3	37
Pst I	3	37
Sma I	4	25
Hind III	2	37
Spe I	2	37
Sph I	2	37

Restriction enzymes buffers (all from GibcoBRL)

Reaction buffer	pH	Tris-HCl	$MgCl_2$	NaCl	KCl
2	8.0	50 mM	10 mM	50 mM	-
3	8.0	50 mM	10 mM	100 mM	-
4	7.4	20 mM	5 mM	-	50 mM

2.1.7. Expression plasmids

The expression plasmid ORF9-hCASP8b contained the full-length cDNA of caspase-8 under the control of the EF1α/HTLV promoter (Cayla-InvivoGen).

The following expression plasmids contained full-length cDNA under control of a CMV promoter: caspase-10 (pCMV-SPORT6-caspase-10; RZPD), DR4 (pCMV-SPORT6-DR4; RZPD) and DR5 (pCMV-SPORT6-DR5; RZPD).

2.1.8. Kits

Endofree Plasmid Extraction Kit (for plasmid preparation)	Qiagen
Cell Death Detection Kit (for quantification of apoptosis)	Roche
Cytotoxicity Detection Kit (for quantification of cell toxicity)	Roche
TransAM NF-κB Family Kit (for quantification of NF-κB activation)	Active Motif
Nuclear Extract Kit	Active Motif
BCA Protein Assay Kit (for protein quantification)	Pierce

2.1.9. DNA and protein markers

DNA-Marker (Gibco)

Fragments in bp: 12216 , 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 506/517, 396, 344, 298, 220, 201, 154, 134, 75.

Protein-Markers

Prestained SDS-PAGE Standard (Broad Range)	BioRad
Molecular Weight Marker	Santa Cruz

2.1.10. Extraction buffers for cellular proteins

Lysis buffer (Bayer):

10 mM Tris-HCl, pH 7.5

144 mM NaCl

1% Nonidet P-40

0.5% SDS

Protease-Inhibiters

1 mM EDTA

2 mM PMSF

10 µg/ml Trasylol

20 µM leupeptin

10 µM pepstatin

CHAPS lysis buffer (for detection caspases cleavage products) (New England Biolabs)

50 mM PIPES/HCl, pH 6.5

2 mM EDTA

0.1% CHAPS

40 µM leupeptin

15 µM pepstatin

10 µg/ml trasylol

5 mM DTT

2.1.11. Antibodies

Primary antibodies

Antigen	Source	Dilution	Company
TRAIL-R1/DR4	rabbit	1:500	Acris Antibodies
TRAIL-R2/DR5	rabbit	1:500	Acris Antibodies
Caspase-8	mouse	1:1,000	New England Biolabs
Caspase-10	mouse	1:1,000	MBL International
Caspase-9	rabbit	1:1,000	New England Biolabs
Caspase-3	rabbit	1:1,000	New England Biolabs
Caspase-7	rabbit	1:1,000	New England Biolabs
BID	rabbit	1:1,000	New England Biolabs
DFF45	rabbit	1:250	New England Biolabs
XIAP	rabbit	1:1,000	New England Biolabs
Bcl-2	mouse	1:200	Santa Cruz Biotechnology
Bcl-x_L	rabbit	1:200	Santa Cruz Biotechnology
Survivin	rabbit	1:200	Santa Cruz Biotechnology
FLIP	mouse	1:200	Santa Cruz Biotechnology
Livin/ML-IAP	mouse	1:1,000	Active Motif
β-actin	mouse	1:10,000	Sigma-Aldrich

Secondary antibodies:

horseradish peroxidase-labelled goat anti-rabbit (1:5,000) Dako Cytomation

horseradish peroxidase-labelled goat anti-mouse (1:5,000) Dako Cytomation

Specific antibodies for detecting NF-κB subunits (Active Motif):

Subunits	Source	Dilution
p65	rabbit	1:1,000

p50	rabbit	1:1,000
c-Rel	rabbit	1:1,000
Rel-B	rabbit	1:1,000
p52	rabbit	1:1,000

Specific antibodies for Flow Cytometry (Alexis)

Antigen	Source	Dilution
TRAIL-R1/DR4	mouse	1:100
TRAIL-R2/DR5	mouse	1:100
IgG_1- (control)	mouse	1:100
mouse IgG_1 (PE-labelled)	goat	1:100

2.1.12. Solutions

10 x TBS (Tris-Buffered Saline)

24.2 g Tris-Base
80 g NaCl
pH 7.6
up to 1 L in sterile water

4 x TBE (Tris-Borate EDTA), pH 7.4

0.356 M Tris-Base
0.356 M boric acid
0.01 M EDTA

10 x Klenow-Buffer, pH 7.6

0.5 M Tris HCl
0.1 M $MgCl_2$

50 x TAE (Tris-Acetat EDTA):

242 g Tris-Base
57.1 ml acetic acid
100 ml 0.5 M EDTA (pH 8.0)
up to 1 L in sterile water

10% SDS:

100 g SDS
up to 1 L in sterile water, pH 7.2

0.5 M EDTA:

186.1 g EDTA
up to 1 L in sterile water, pH 8.0

2 x Binding buffer, pH 7.9

40 mM Hepes
120 mM KCl
8% Ficoll

2.1.13. Chemical and radioactive substances

Acrylamid/Bisacrylamid (37.5 : 1)	BioRad
Agarose	Gibco BRL
Ammoniumpersulfat (APS)	BioRad
Bromphenolblue	BioRad
EDTA	Sigma
Ethanol	J.T. Baker
Ethidiumbromide	Sigma
Ficoll	Pharmacia
Formaldehyde	J. T. Baker
Glycerol	Sigma
Glycin	Serva
Isopropanol	J.T.Baker
Methanol	J.T. Baker
N-Acetyl-Leu-Leu-Nle-CHO (LLnL)	Calbiochem
Natriumchlorid	Merck
Natriumcitrate	Merck
Natriumhydroxide	Merck
Non-fat dry Milk	BioRad
Poly [d(I-C)]	Boehringer
Ponceau S	Sigma
Sodium dodecyl sulfat (SDS)	Amresco
TEMED	BioRad
Tris(hydroxymethyl-)aminomethan	Merck, Invitrogen
Tween-20	Calbiochem

Xylene cyanol	BioRad
β-Mercaptoethanol	Merck

Radiochemicals:

alpha ^{32}P-dCTP (6000 Ci/mmol) PerkinElmer

2.1.14. Equipment

centrifuges	Biofuge pico, Heraeus
	Biofuge fresco, Heraeus
	Laborfuge 400E, Heraeus
	Varifuge RF, Heraeus
	Centrifuge J2-21, Beckman Instruments
imaging densitometer	Model GS-700, Bio-Rad
CO_2 -incubator	Heraeus
drying oven	Heraeus
electrophoresis -equipment	Mini Protean II, Bio-Rad
electrophoresis-power supplies	Model 1000/500, Bio-Rad
ELISA photometer	Model 550, Bio-Rad
flow cytometry system	FACSCalibur, BD Biosciences
heating bath	1.002, GFL
laminar flow bench	BSB 4A, Gelaire Flow Laboratories
magnetic stirrer	MR 2000, Heidolph
microscopes	Diavert, Leitz
	BX60F5, Olympus
pH- meter	pH 526, WTW
precision balances	Kern 474, Gottl Kern& Sohn
	Type 2662, Sartorius
western blot transfer equipment	Mini-Trans-Blot, Bio-Rad
BAS-1500 Phosphoimager	Fuji Photo

2.2. Methods

2.2.1. Cultivation of cells

Melanoma cells were grown in tissue culture flasks (75 cm^2) with 12 ml DMEM/10% FCS, in a humidified atmosphere with 5% CO_2 at 37 °C. All melanoma cell lines grew adherent on the ground of the flasks. Every third day the growth medium was removed. For passaging, 80% confluent cells were rinsed with PBS, and then were incubated with 1 ml trypsin solution at 37 °C until detachment of the cells from the flask's ground was seen (3-5 minutes). Cells were harvested in 10 ml DMEM with 10% FCS to stop the proteolytic activity of trypsin. The cells were then pelleted at 200 x g for 5 minutes. After aspiration of the supernatant, the cells were resuspended in DMEM/10% FCS, and distinct numbers of cells were distributed into new flasks. The first change of medium occurred after 36 hours.

2.2.2. Freezing and thawing of cells

70% confluent cells, which received fresh growth medium the day before, were washed with 10 ml PBS and trypsinized as described previously (2.2.1). The cells were then pelleted at 200 x g for 5 minutes. After aspiration of the supernatant, the cells were resuspended in a freezing solution containing 10% DMSO and 25% FCS. The cell suspension was then transferred into 1 ml cryotubes. The presence of DMSO in the freezing solution prevents the formation of ice crystals within cells. Freezing of cells has to be a slow process, therefore the freezing tubes were wrapped up in a tick heap of paper and kept overnight in a refrigerator at -80 °C. The day after, stock tubes were transferred to a container with liquid nitrogen (-196° C) for long term storage.

In contrast, thawing of the cells should be fast. Therefore, cells were thawed in a 37 °C water bath and resuspended in 10 ml of warm DMEM/10% FCS. The cells were then pelleted by centrifugation at 200 x g for 5 minutes. After aspiration of the supernatant, a cell pellet was resuspended in 12 ml culture medium and was then redistributed in a culture flask (75 cm^2).

2.2.3. Detection of apoptosis

Apoptosis can be assessed by various methods, among which the measurement of DNA fragmentation is highly reliable. DNA fragmentation, representing the cleavage of chromosomal DNA in oligonucleosome-sized fragments, is a biochemical hallmark of apoptosis. A specific endonuclease (CAD), which is activated at the end of the apoptosis signalling cascade, cleaves the nuclear DNA at accessible internucleosomal sites resulting in

the release of mono- or oligonucleosomes in the cytoplasm. Released DNA fragments are therefore discrete multiples of a 180 bp subunit. They remain bound to histones and can be detected using a combination of anti-histone and anti-DNA antibodies. Detection of mono and oligonucleosomes in the cytoplasm is possible due to occurrence of DNA fragmentation several hours before plasma membrane breakdown. Here, DNA fragmentation was measured using a commercially available kit "Cell Death detection ELISA plus" from Roche Diagnostics. The test was performed on streptavidin-coated microtiter plate. After transfer of cell lysates to the microtiter wells, a mixture of anti-histone biotin-antibodies and peroxidase-coupled anti-DNA antibodies was added. The anti-histone antibodies recognised the protein fraction of the nucleosomes and immobilised it via streptavidin-biotin interaction on the microtiter plate. The DNA coiled around histones was recognised by the peroxidase-coupled anti-DNA antibodies. A chromogenic peroxidase substrate was added to measure the absorption by an ELISA photometer.

Reagents used (included in the kit):

anti-histone-antibodies, coupled with biotin
anti-DNA-antibodies, coupled with peroxidase
incubation buffer
lysis buffer
positive control
substrate solution (2,2'-azino-di[3-ethylbenzthiazoline-sulfonate])

immunoreagent solution for 30 probes:

120 µl of anti-histone-antibodies
120 µl of anti-DNA-antibodies
2160 µl of incubation buffer

Protocol:

For induction of apoptosis, melanoma cells were treated either with *killer*TRAIL at concentrations of 20 ng/ml and 100 ng/ml, with CH-11 (500 ng/ml) or with TNF-α (10 ng/ml) for different lengths of time. Cells were seeded in 6-well plates ($2x10^5$ cells/well), and the day after, growth medium was replaced by 2 ml medium containing either TRAIL, CH-11 or TNF-α. After incubation, plates were centrifuged at 200 x g for 5 min. The supernatant was removed, and cells were lysed in lysis buffer (1 ml/well) for 30 min at room temperature wile being subjected to moderate shaking. A cytosolic extract was prepared by centrifugation of plates at 300 x g for 10 min. Subsequently, 5µl of the supernatant with 15 µl of lysis buffer was added to streptavidin-coated microtiter plates. The probes are then incubated for 2h with

80µl of the immunoreagent solution at room temperature wile being subjected to moderate shaking. After washing, each well received 100µl substrate solution. Incubation at room temperature was performed until specific colour reaction appeared in the positive control (usually 10 min). Absorption at 409 nm was measured using an ELISA photometer. Relative apoptosis rates determined after treatment with TRAIL were calculated with respect to untreated control cells, which were set to 1.

2.2.4. Cytotoxicity assay

Cell necrosis is characterised by plasma membrane damage and release of the intracellular content in the surrounding medium. Cell death can be evaluated by measuring the uptake or exclusion of vital dyes (such as trypan blue, eosin Y, nigrosine, propidium iodide or ethidium bromide), the release of radioactive isotopes from previously labelled cells or the activity of cytoplasmatic enzymes which are released upon cell death. Lactate dehydrogenase (LDH) is a relatively stable cytoplasmatic enzyme, present in almost all cells, which is rapidly released in the culture supernatant after damage of the plasma membrane. LDH activity was evaluated with the "Cytotoxicity Detection Kit (LDH)" from Roche Diagnostics (Mannheim, Germany), by means of a two-step enzymatic reaction. In the first step, NAD+ is reduced to NADH/H+ by the LDH-catalysed oxidation of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to the tetrazoliumsalt 2-[4-iodophenyl]-3-[4-nytrophenyl]-5-phenyltetrazolium chloride, which is then reduced to red coloured formazan. The increase in LDH enzyme activity following plasma membrane cell damage correlates with the amount of formed formazan which can be measured by an ELISA photometer at 490 nm. The cell culture supernatant can be stored for a few days at 4^0C without any changes of LDH enzyme activity. The increase in LDH activity in the supernatant is proportional to the quantity of damaged cells.

Reactive mixture:

catalyst-solution 2,5 µl
dye solution 118 µl

Protocol:

Cells were seeded at $2x10^5$ cells/well in 6-well plates and allowed to adhere overnight. The day after, they were treated with apoptosis inducing agents. The cell supernatant was then collected and centrifuged at 200 x g for 5 minutes. Then, 100 µl of the resulting supernatant was transferred into a microtiter plate and mixed with 100 µl of reaction buffer. The plate

with samples was wrapped in light protecting paper and incubated for 30 min at room temperature. The absorption at 490 nm was measured by an ELISA photometer. Relative cytotoxicity rates determined after treatment with TRAIL were calculated with respect to untreated control cells, which were set to 1.

2.2.5. Protein analysis

2.2.5.1. Determination of protein concentration

Protein concentration was determined using a commercially available kit from Pierce (Weiskirchen). The principle of the method is the biuret reaction, where the amino group of proteins reduces copper (II) - cations to copper (I)-cations in alkaline medium. Copper(I)-cations then react with two molecules of bicinchoninic acid (BCA) and a stable chelate complex of violet color rises, which exhibits maximal absorption at 562 nm. Depending of the incubation temperature, different sensitivities can be achieved:

Incubation time	**t [°C]**	**Concentration range**
30 min	60	5-250 µg/ml
30 min	37	20-1.200 µg/ml
2 h	room temperature	20-1,200 µg/ml

Solutions used:

Solution A:

Components	**Concentration**	**Quantity**
disodium bicinchoninate	26 mM	10 g
disodium carbonate-monohydrate	161 mM	20 g
disodium tatrate-dihydrate	7 mM	1.6 g
NaOH	100 mM	4 g
sodium hydrogen carbonate	113 mM	9.5 g
water, double distilled		up to 1,000 ml

Solution B:

Components	Concentration	Quantity
copper II sulphate-pentahydrate	160 mM	1 g
water, double distilled		up to 25 ml

Protocol:

The working reagent was prepared by mixing fifty parts of Solution A to one part of Solution B. A protein standard rank was prepared by successive dilutions of bovine serum albumin (BSA) in sample buffer. Sample buffer contains usually leupeptin and pepstatin, which are peptides and therefore also undergo the biuret reaction. Further components of the sample buffer such as phenylmethanesulfonyl fluoride (PMSF) and dithiothreitol (DTT) act in change as reducing agents. Protein samples were also diluted in sample buffer (1:5). Each protein sample (10 µl) and the standard rank probes were transferred to a microtiter plate in triplicates. Working solution (200µl/well) was added and the probes were further incubated at the appropriate temperature. The absorption was then measured at 570 nm with an ELISA photometer. The protein concentration was calculated using the standard rank curve.

2.2.6. SDS-polyacrylamide gel electrophoresis

The discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Analytical electrophoresis of proteins was carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Polyacrylamide gels were prepared by free radical-induced polymerization of acrylamide with N,N'-methylene bisacrylamide as cross-linker. Ammonium persulphate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED) were added as provider and stabilizer of free radicals, respectively.

The strong anionic detergent SDS was used in combination with heat to dissociate proteins before they were loaded on the gel. Dithiothreitol (DTT) was added to the sample buffer to reduce disulphide bonds. The amount of SDS bound is nearly proportional to the molecular weight of the polypeptide (about 1.4g of SDS per 1g of protein), and independent of its sequence. The large negative charge of SDS masks the intrinsic charge of proteins, so that complexes of identical charge-to-mass ratio are formed. In consequence, SDS-polypeptide complexes migrate through polyacrylamide gels according to the size of the polypeptide. The

molecular weight of unknown polypeptide chains can be estimated by running in parallel markers of known molecular weight.

Solutions:

AA-Bis

Components	**Concentration**	**Quantity**
acrylamide	5.44 M	386.6g
N,N-methylenebisacrylamide	87mM	13.4g
water, double-distilled		up to 1,000ml

10 % APS

Components	**Concentration**	**Quantity**
ammonium persulphate	0.44 M	0.1 g
water double-distilled		up to 1 ml

Running Gel Buffer (5x)

Components	**Concentration**	**Quantity**
Tris	375 mM	113.5 g
sodium dodecyl sulphate	35 mM	2.5 g
hydrochloric acid		ad pH 8.8
water, double-distilled		up to 500 ml

Stacking gel Buffer (4x)

Components	**Concentration**	**Quantity**
Tris	175 mM	16.95 g
sodium dodecyl sulphate	35 mM	0.80 g
hydrochloric acid		ad pH 6.8
water, double-distilled		up to 200 ml

Electrophoresis Buffer (10x) (SDS)

Components	**Concentration**	**Quantity**
Tris	175 mM	60.55 g
sodium dodecyl sulphate	35 mM	20.19 g
glycine	190 mM	285.27 g
water, double-distilled		up to 200 mL

Loading Buffer (5x)

Components	**Concentration**	**Quantity**
1 M Tris/ HCl pH 6.8	30 mM	750 µl
glycerol	0.7 M	1 250 µl
sodium dodecyl sulphate	35 mM	0.25 g
1,4-dithiothreitol	0.1 M	0.39 g
1 % bromophenol blue	0.14 mM	250 µl
water, double-distilled		up to 5 ml

Pipetting scheme for 2 gels, 80 mm x 60 mm x 1.5 mm in size:

Components	**Running gel**					**Stacking gel**
	5 %	7.5 %	10 %	12.5 %	15 %	3 %
water double-distilled [ml]	13.4	12.5	10.9	9.65	8.4	5.34
5x running gel buffer [ml]	4	4	4	4	4	-
4x stacking gel buffer [ml]	-	-	-	-	-	2.0
acrylamid (40 %) [ml]	2.5	3.75	5.0	6.25	7.5	0.6
TEMED [µl]	5	5	5	5	5	6
10 % APS [µl]	95	95	95	95	95	55

Protocol:

The running gel solution was prepared according to the scheme given above. Polymerisation was started by adding TEMED and ammonium persulphate (APS). The solution was carefully mixed and poured into the gap between two glass plates of a slab gel unit (Bio Rad). Then the running gel was cautiously overlaid with water-saturated 1-butanol. After completion of the polymerization (30-45 min), the overlay was poured off preceding complete removal with the edge of a Whatman 3 MM paper. The stacking gel solution was then poured directly onto the

surface of the polymerized running gel, and a clean plastic comb was inserted immediately, avoiding the formation of air bubbles. After complete polymerization, the comb was removed and the slab gel unit was inserted into the electrophoresis chamber filled with electrophoresis buffer. The samples were adjusted to equal protein concentrations through dilution in the corresponding lysis buffer.

Protein samples received one fourth of their volume loading buffer (5x). Proteins are then denaturated by incubation at 95°C and loaded with a Hamilton syringe to the stacking gel wells. Electrophoresis was performed at 80V for 15 min, and subsequently at 120V. When the bromphenol blue had reached the bottom of the running gel, electrophoresis was stopped. The gel slab units was disassembled and prepared for Western blotting.

2.2.7. Western blotting

Different proteins separated by SDS-PAGE can be identified by binding specific antibodies. An antigen-antibody reaction cannot properly occur inside the polyacrylamide gel, so that the proteins have to be first transferred to a nitrocellulose membrane before exposure to the antibodies. The transfer of proteins to the membrane was performed directly after electrophoresis. The proteins were negatively charged at the transfer pH, due to the bound SDS, so they would migrate from the cathode to the anode.
The membrane loaded with proteins was blocked with 5% non-fat milk for 1 h to prevent unspecific binding and was then incubated with the specific antibody. The bound antibodies were subsequently labelled with secondary anti-IgG antibodies coupled with horseradish peroxidase, which allowed the visualisation of the protein-antibody complexes on addition of a chromogen substrate.

Solutions used:

Transfer buffer (blotting buffer) (2x)

Components	**Concentration**	**Quantity**
Tris	25 mM	12.11 g
glycine	192 mM	57.65 g
methanol	4.9 M	800 ml
water double-distilled		up to 2,000 ml

The pH should be 8.3 without adjusting.

PBS (10x)

Components	Concentration	Quantity
Potassium chloride	2.68 mM	4 g
Potassium dyhidrogen phosphate	1.47 mM	4 g
Sodium chloride	137 mM	160 g
Disodium hydrogenphosphate dihydrate	8.09 mM	28.8 g
water, double-distilled		up to 2,000 ml

pH should be 7.4 and should be not adjusted. Osmolarity is about 300 mosmol.

Staining solution:

Components	Concentration	Quantity
Ponceau S	13 mM	10 g
Glacial acetic acid	0.9 M	50 ml
water, double-distilled		up to 1,000 ml

Protocol:

Blotting followed directly according to SDS-PAGE. Whatman papers and nitrocellulose filters were cut to the size of the gels and soaked in transfer buffer for 5 min. The transfer sandwich was assembled adjacent to the cathode in the following order: a porous pad, two pieces of 3 MM paper, the gel, the blotting membrane, and another two pieces 3 MM paper. Air bubbles were removed by rolling a glass pipette over the sandwich. A last porous pad was placed on the top, the cassette was folded and inserted into the blotting chamber, with the membrane towards to the anode. The chamber was filled with transfer buffer to completely cover the electrode panels. Having placed a magnetic stirrer for mixing the buffer, the chamber was placed on ice for cooling during the transfer. The blotting was performed for 1.5h at 100V. The successful transfer of proteins onto the membrane was proved by staining the membrane with Ponceau solution, which provided a transient staining of protein bands and did not interfere with the subsequent antibody binding. The filters were incubated for 2-5 min with Ponceau S wile being subjected gentle shaking at room temperature. When the protein bands became visible, the filters were washed several times in double distilled water and prepared for immunodetection of proteins.

2.2.7. Immunodetection of blotted proteins

Membranes were incubated with the respective primary antibodies. Subsequently, they were incubated with the secondary antibodies, specific for the common structure of the primary antibodies. The secondary antibodies were coupled to peroxidase to produce a chemiluminescent signal. Peroxidase can reduce hydrogen peroxide to water, using luminol as reducing agent. An enhancing agent further stabilised the excitation state of luminol acquired by reduction. Upon returning to the original state of luminol, light is emitted and can be detected on X-ray films.

Solutions used:

PBS-T (1x)

Components	Concentration	Quantity
Tween-20	0.05 %	500 µl
PBS 10 x		50 ml
water, double distilled		up to 1,000 ml

Blocking buffer

Components	Concentration	Quantity
Skim milk powder	5 %	5 g
PBS-T (1x)		100 ml

Super Signal working solution

Components	Quantity
Luminol/ enhancer solution	5 ml
stable peroxide solution	5 ml

The working solution was stable for approximately 24 h. The luminol/ enhancer solution and the stable peroxide solution were purchased from Perbio. The supplier did not provide information about the ingredients.

Protocol

After staining with Ponceau S, the membranes were washed 2-3 times in doubled distilled water, and were then incubated for 1 h in blocking buffer (10 ml/membrane), at room temperature wile being subjected moderate shaking. The blocking buffer was then removed,

and 10 ml blocking buffer containing primary antibodies was added to the membranes. Incubation lasted either 1 h at room temperature or overnight at 4°C wile being subjected gentle agitation. Subsequently, the membranes were washed 3 times for 5 min in 0.05% PBS/Tween-20 and were then incubated with the secondary antibodies diluted in blocking buffer as indicated for 1 h at room temperature wile being subjected gentle agitation. The solution with secondary antibodies was discarded and the membranes were again washed 3 times in 0.05% PBS/Tween-20 for 5 min each time and finally once with PBS only. For the chemiluminescence reaction, the membranes were incubated for 1 min in Super Signal working solution at room temperature. The membranes were then placed in a protective plastic sheet, excess of substrate solution and air bubbles were removed by gentle pressing, and then the membranes together with an X-ray film were enclosed in a film exposure cassette. The duration of the film exposure varied according to the intensity of the signal.

2.2.9. Proteasome inhibition and in vitro Viability Assay

Viability assays could be determined as an alternative method to apoptosis assays described above. The principle of this method implies the estimation of the number of surviving cells after treatment with an apoptosis stimulus. Surviving cells were attached to the ground of culture flasks, whereas apoptotic cells could be washed away. According to this method, after washing, the adherent cells were fixed with glutaraldehyde and then stained with crystal violet. The bound dye can then be released from cells by lysis with Triton-X100 that later is measured using the ELISA photometer.

Solutions:

Fixing solution

Components	**Concentration**	**Quantity**
50 % Glutaraldehyde	0.1 M	250 µl
PBS		12.25 ml

Crystal violet stock solution

Components	**Concentration**	**Quantity**
crystal violet	2 mM	15 mg
PBS		15 ml

Destaining solution

Components	Concentration	Quantity
Triton X100	0.2 %	10 µl
PBS		5 ml

Protocol:

Cells were seeded in 24-well plates with 5x 10^4 cells/well. After 24 h, growth medium was replaced by 0.25 ml medium containing the proteosome inhibitor N-Acetyl-Leu-Leu-Nle-CHO (LLnL, 5 µM; Calbiochem). After another 2 h, cells were treated with TRAIL (100 ng/ml) for an additional 24 h. After incubation, supernatant was removed, cells were rinsed in 250 µl PBS/well and then were fixed with 500 µl/well of the fixing solution over 30 minutes, at room temperature wile being subjected moderate shaking. The fixing solution was then aspirated and the cells were washed with PBS (500 µl/well). Staining was performed by adding 500 µl of dye solution diluted in 1:10 in PBS to each well and incubating for another 30 minutes at room temperature wile being subjected moderate shaking. Unbound dye was then washed away by submerging the plates in deionised water for 15 minutes. After washing, the water excess was removed from the plates by tapping on tissue paper. To release the bound dye, 200 µl of distaining solution was added to each well, and incubation was performed for at least 1 h at room temperature wile being subjected moderate shaking. After incubation, 100 µl of supernatant from each well was transferred into a microtiter plate and the extinction was measured at 570 nM using an ELISA photometer. Relative survival rates determined after treatment with TRAIL and LLnL were calculated with respect to untreated control cells, which were set to 100%.

2.2.10. Fluorescence-activated cell sorting (FACS) analyses

The term "Flow Cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. The fundamental concept is that cells flow one at a time through a region of examination where multiple biophysical properties of each cell can be measured at rates of over 1000 cells per second. To enable the measurement of biological properties, cells are usually stained with fluorescent dyes, which bind specifically to cellular constituents. The dyes emit light at a long wavelength after excitation by a laser beam. The emitted light is picked up by detectors, and the signals are digitalized so that they may be stored for later display and analysis. Biological targets can be stained either directly by

specific antibodies, which contain fluorescent dyes, or indirectly by using secondary labeled antibodies.

This method is the choice for measuring surface expression of TRAIL death receptors.

Solutions used:

Detaching solution

Components	**Concentration**	**Quantity**
EDTA	0.5 M	50 µl
PBS		50 ml

Washing solution

Components	**Concentration**	**Quantity**
BSA	1%	500 mg
PBS		50 ml

Fixation solution

Components	**Concentration**	**Quantity**
paraformaldehyd	1%	300 mg
PBS		30 ml

Protocol:

Cells were grown in culture flack (75 cm^2) for two days and before the examination the old medium was replaced by the fresh. The day after, cells were detached with EDTA/PBS for 10 min for avoiding unintentional damage of cells by trypsin/EDTA. Detached cells were collected with PBS in 50 ml tube and centrifuged at 200 x g for 5 min. After washing with 1% BSA/PBS, aliquots of $5x10^5$ cells in 100 µl 1% BSA/PBS were incubated for 30 min with mAb against TRAIL-R1/DR4 (Alexis, 1:100) or with mAb against TRAIL-R2/DR5 (Alexis, 1:100). The isotypic monoclonal mouse IgG_1 antibody (Alexis, 1:100) was used as a negative control. After incubation, cells were washed two times with 1% BSA/PBS. After that, they were incubated for 30 min with the secondary phycoerythrin-labeled goat anti-mouse IgG_1 antiserum (Alexis, 1:100). Cells were washed once with 1% BSA/PBS and with PBS and then fixed with 1% paraformaldehyde. Surface expression of TRAIL death receptors was determined with a FACS-Calibur™ flow cytometer (Becton Dickinson). The mean fluorescence index was calculated by using CellQuest™ software (Becton Dickinson).

2.2.11. Determination of NF-κB activity

The transcription factor NF-κB is implicated in the regulation of many genes that code for mediators of the immune and inflammatory responses as well as factors leading to tumor development, angiogenesis, metastasis and apoptosis resistance. NF-κB is composed of homo- and heterodimeric complexes of members of the Rel (NF-κB) family. There are five subunits of the NF-κB family: p50, p65 (RelA), c-Rel, p52 and RelB3. In the majority of cells, NF-κB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins. Release of NF-κB from their natural inhibitors leads to their translocation into the nucleus, where they activate appropriate target genes.
The electrophoretic mobility shift assay (EMSA) as well as ELISA are appropriate methods for determining NF-κB nuclear translocation and activation.

2.2.11.1. Determination of NF-κB activation by ELISA

TransAM NF-κB Kit (Activ Motif) contains a 96-well plate on which oligonucleotides containing the NF-κB consensus site (5′-GGGACTTTCC-3′) have been immobilized. The active NF-κB subunits in nuclear extracts specifically bind to the oligonucleotides. The primary antibody used for detection of NF-κB recognizes an epitope common to all NF-κB subunits (p50, p52, p65, c-Rel or RelB), which is accessible only when NF-κB is activated and is bound to the target DNA. An HRP conjugated secondary antibody provides a sensitive colorimetric readout that is quantified using spectrophotometer. As this assay is performed in 96-well plates, a number of samples can be handled simultaneously.

Solutions:

Complete Lysis Buffer (for one well)

Components	**Quantity**
DTT	0.11 µl
Protease inhibitor cocktail	0.23 µl
Lysis Buffer AM2	22.2 µl

Complete Binding Buffer (for one well)

Components	Quantity
DTT	0.07 µl
Herring sperm DNA	0.34 µl
Binding Buffer AM3	33.4 µl

Complete Binding Buffer with wild-type or mutated oligonucleotide (for one well)

Components	Quantity
Wild-type or mutated oligo	2 µl
Complete Binding Buffer	31.8 µl

1X Washing Buffer (for one well)

Components	Quantity
Distilled water	2,025 ml
10X Washing Buffer AM2	225 µl

1X Antibody Binding Buffer (for one well)

Components	Quantity
Distilled water	202.5 µl
10X Ab Binding Buffer AM2	22.5 µl

Nuclear extraction

Solutions used:

PIB (Phosphatase Inhibitor Buffer) (for 10 ml)

Components	Concentration	Quantity
NaF	125 mM	52 mg
β-glycerophosphate	250 mM	0.55 g
para-nitrophenyl phosphate (PNPP)	250 mM	1.15 g
$NaVO_3$	25 mM	31 mg

HB (Hypotonic Buffer)

Components	Concentration	Quantity
Hepes, pH 7.5	20 mM	0.24 g
NaF	5 mM	12 mg
Na_2MoO_4	10 µM	5 µl of a 0.1 M solution
EDTA	0.1 mM	10 µl of a 0.5 M solution

Protocol:

Melanoma cells (2x 10^6) were seeded in culture plates (50 cm^2) and treated with TRAIL or with TNF-α (varying times and concentrations). For blocking experiments, cells were pretreated with blocking antibodies against TRAIL-R1/DR4 (Alexis, 1:100) TRAIL-R2/DR5 (Alexis, 1:100) for 1h. Nuclear extracts were prepared with a nuclear extraction kit (Active Motif). The protein content in nuclear extracts was quantified by the BCA protein assay (Pierce, Bonn, Germany). Equal amounts (7 µg) of nuclear proteins were used. There are four steps for performing this assay, which are described below.

Step 1: Binding of NF-κB to its consensus sequence

Complete binding buffer (30 µl) was added to each well to be used. For performing competitive binding experiments, 20 pmol (2 µl) of the wild-type or mutated consensus oligonucleotide were added. Nuclear protein extracts (7 µg) were diluted in complete lysis buffer to a volume of 20 µl, which was added to the respective well. Incubation was performed for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform). Afterwards, wells were washed 3-times with 200 µl washing buffer.

Step 2: Binding of primary antibody

After discarding the last washing buffer, the diluted NF-κB antibodies (1:1.000 dilution in antibody binding buffer, 100 µl/well) were applied to each well. The plate was incubated for 1 h at room temperature without agitation. Wells were then washed again 3 times with 200 µl washing buffer.

Step 3: Binding of secondary antibody

HRP-conjugated antibody (1:1,000 dilution in antibody binding buffer, 100 µl/well) was applied to the wells. Incubation lasted for 1 h at room temperature without agitation. Wells were washed 4-times with 200 µl washing buffer.

Step 4: Colorimetric reaction

The developing solution (100 µl/well) was added to each well, and incubation was performed at room-temperature for 2-10 min. The colour reaction was monitored until the developing solution turned blue. At the end, 100 µl/well stop solution were added, which turned the blue colour to yellow. The chromogenic reaction was measured at 450 nm with a reference wavelength of 655 nm in an ELISA reader.

2.2.11.2. Electromobility shift assay

The DNA-binding activity of NF-κB can be determined by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this method, nuclear extracts are incubated with a radioactively labelled double-stranded oligonucleotide probe containing the consensus sequence for NF-κB binding. Samples are then separeted by electrophoresis on native polyacrylamide gels, followed by drying the gel and autoradiography. This method is considered as a standard technique for measuring NF-κB activity however, it is more time consuming (several days of gel exposure may be required to achieve sufficient sensitivity) and it cannot be applied to high-throughput screening.

Solution used

5% Acrylamide gel

Components	**Quantity**
4 x TBE	20.0 ml
30% Acrylamidgel	13.30 ml
10% APS	600 µl
TEMED	60 µl
Bidistilled water	46.04 ml

NF-κB – Oligo (0.02 µmol) (Metabion)

Sense 5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3'

Antisense 3'-AGTCTCCCCTGAAAGGCT CTCCAGCT-5'

DNA-Radiolabelling

Components	**Concentration**	**Quantity**
10 x Klenow buffer		2.5 µl
ds Oligo	100 pmole/µl	0.5 µl
dNTPs	0.5 mM	1.8 µl
α ^{32}P-dCTP	6,000 Ci/mmol	1.5 µl
Klenow Fragment	3 U/µl	1.0 µl
H_2O		17.7 µl

Binding mix

Components	**Quantity**
Nuclear extracts	7 µg
2 x Binding buffer	10 µl
DTT	0.4 µl
BSA	0.2 µl
Poly [d(I-C)]	1 µl
^{32}P-dCTP Oligo	10^5 cpm
H_2O	up to 20 µl

Protocol:

Two complementary oligonucleotides covering the NF-κB consensus sequence were used: 5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3' (sense) and 3'-AGTCTCCCCTGAAAGGCTCTCCAGCT-5' (antisense). The annealed oligonucleotides were radiolabeled by filling up the overhanging ends with ^{32}P-dCTP (PerkinElmer) by Klenow polymerase (Invitrogen). Binding reactions were carried out at room temperature for 30 min. For one reaction, 7 µg of nuclear extract and 10^5 cpm of ^{32}P-labeled, double-stranded oligonucleotide were applied in 20 µl binding buffer (20 mM Hepes, 60 mM KCl, 4% Ficoll, pH 7.9, 2 mM DTT, 100 µg/ml BSA and 100 µg/ml poly d(I-C)). Samples were analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels using 0.5x TBE buffer, pH 7.4.

Dried gels were exposed to an imaging plate for quantification of radioactivity by a BAS-1500 Phosphoimager (Fuji Photo). Supershift controls were performed by using antibodies specific for NF-κB subunits p50 and p65 (sc-1191; sc-109; Santa Cruz Biotechnology).

2.2.12. Restriction analysis

Reagents used

Mixture for plasmid restriction

Components	**Quantity**
DNA plasmid	0.4 µg
10x Buffer	2 µl
10x BSA	2 µl
Enzyme	0.5 µl
Distilled water	up to 20 µl

Combination of enzymes

caspase-8 (ORF9-hCASP8b; Cayla-InvivoGen)

Restriction enzymes	**Buffer**	**10x BSA**	**t [°C]**
EcoR I/Hind III	2	-	37
Nco I/Hind III	2	-	37
Pst I/Xho I	3	+	37

caspase-10 (pCMV-SPORT6-caspase-10; RZPD)

Restriction enzymes	**Buffer**	**10x BSA**	**t [°C]**
Hind III	2	-	37
Nco I	2	-	37
Spe I	2	+	37
Sph I	2	-	37
Sph I/Nco I	2	-	37
EcoR V/Not I	3	+	37
EcoR I/Not I	3	+	37

DR4 (pCMV-SPORT6-DR4; RZPD)

Restriction enzymes	**Buffer**	**10x BSA**	**t [°C]**
Sal I/Not I	3	+	37
Sal I/Xba I	3	+	37
Sal I/Xba I/Apa I	4 / 3	+	37 / 25
Nco I/Apa I	4	+	37 / 25

DR5 (pCMV-SPORT6-DR5; RZPD)

Restriction enzymes	**Buffer**	**10x BSA**	**t [°C]**
Sal I/Not I	3	+	37
Sal I/Xba I	3	+	37
Nco I/Sma I	4	+	37 / 25
Nco I/Sac I	4	+	37

1% Agarose gel

Components	**Quantity**
Agarose	0.6 g
50x TAE buffer	12 ml
Ethidium bromide (10 mg/ml)	5 µl
Bidistilled water	600 ml

Protocol:

The agarose was filled up with distilled water, heated in a microwave oven until all agarose was dissolved. After cooling down to 65°C, ethidium bromide was added. The gel was poured into gel tray and left for 1 h until it hardened. The chamber was filled up with 1x TAE so that the gel was completely covered.

In parallel, plasmids containing the gene of interest were prepared for restriction analysis. All components were mixed and incubated for 2 h wile being subjected appropriate conditions.

After mixing with DNA loading buffer (1/5 of the sample volume), samples were loaded into the pockets of the gel. The electrophoresis was run at 100 V (150 mA) until the bromphenol blue marker reached 2/3 of the gel length. The gel stained with ethidium bromide was photographed on a UV-transilluminator using a CCD camera (Polaroid). The mobility of product fragments compared to the mobility of the DNA marker.

2.2.13. Transient transfection

A-375/mock and A-375/Bcl-2 as well as MelHO/mock and MelHO/Bcl-2 have been established by transfection of A-375 and Mel-HO with either pIRES plasmid (Clontech) or with a pIRES construct including a full length cDNA for mouse Bcl-2 (pIRES-Bcl-2), as described previously (Raisova *et al*, 2001). The tetracycline-regulatable melanoma cell line SKM-13-Tet-On were derived from stable transfection of the pTet-On plasmid (Clontech) into SK-Mel-13 melanoma cells. Stable SKM-13-Bcl-x_L and SKM-13-mock cell clones were derived from transfection of SKM-13-Tet-On with pTRE-Bcl-x_L and pTRE-1 (Clontech), respectively, (Hossini *et al*, 2003). Gene expression was induced in these cells by 2 µg/ml doxycycline.

For overexpression of caspases and death receptors, melanoma cell lines were transiently transfected with expression plasmids. An expression plasmid containing the full-length cDNA of caspase-8 under control of a EF1α/HTLV promoter (ORF9-hCASP8b; Cayla-InvivoGen) was used as well as three plasmids containing full-length cDNA under control of a CMV promoter: caspase-10 (pCMV-SPORT6-caspase-10; RZPD), DR4 (pCMV-SPORT6-DR4; RZPD) and DR5 (pCMV-SPORT6-DR5; RZPD).

In brief, cells were seeded into 6-well plates with 2 x 10^5 cells/well. The next day, cells at a confluence of 50% were washed with serum-free Opti-MEM medium (Invitrogen) followed by 4 h incubation at 37°C with 1 ml/well of the respective transfection solution. Transfection solutions consisted of 1 ml/well (6-well plate) of Opti-MEM supplemented with 1µl of DMRIE-C (Invitrogen) and 0.8 µg plasmid DNA (for caspase-8 and caspase-10) or 0.2 µg plasmid DNA (for DR4 and DR5). After transfection, cells were incubated for 48 h in normal growth medium for recovery before they were treated with TRAIL (20 ng/ml, 6 h). Subsequently cells were incubated for additional 48 h in their respective growth medium before analysis.

2.2.14. Immunohistochemistry and Immunocytochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded archival tissue. Melanoma sections of 4–6 µm thickness were fixed on SuperFrost plus slides (Roth, Karlsruhe, Germany) by incubation for 4 h at 60 °C followed by overnight incubation at 37 °C. After removal of paraffin by xylol extractions, sections were rehydrated and finally equilibrated in TRIS-buffered saline, pH 7.5 (TBS). For antigen retrieval, sections were boiled for 3 min in sodium citrate buffer (10 mM, pH 6) using a pressure cooker.

Cytospins were prepared after detachment of adherent cells by incubation with 0.02% EDTA in PBS. Cells were washed 2 x with PBS at 4 °C, and aliquots of 30,000 cells were transferred to SuperFrost plus slides (Roth) by centrifugation at 700 rpm for 5 min (Shandon). Slides were air-dried, and adherent cells were fixed with 3% paraformaldehyde in PBS for 10 min at 4° C, followed by 2x washing in PBS, for each 5 min and air-drying again.

For immunostaining of tissue sections and of cytospins by the alkaline phosphatase/anti-alkaline phosphatase method (APAAP), slides were first equilibrated with TBS for 5 min at room temperature and then incubated in a humidity chamber at room temperature with the following solutions: 1) protein blocking solution (DakoCytomation, Hamburg, Germany) for 30 min; 2) primary antibody solution in antibody diluent (DakoCytomation) for 60 min: TRAIL-R1/DR4 rabbit polyclonal (Acris Antibodies, ab8414, 1:150) or TRAIL-R2/DR5 (Acris Antibodies, ab8416, 1:100); 3) mouse anti-rabbit antibody solution (Dako, M0737) for 30 min, diluted 1:200 in HUSE (87.5% RPMI medium supplemented with 10% heat inactivated FCS, 12.5% human serum); 4) rabbit anti-mouse antibody solution for 30 min (DakoCytomation, Z0259), diluted 1:20 in HUSE, 5) APAAP-complex (DakoCytomation, D0651) for 30 min, diluted 1:50 in RPMI medium supplemented with 10% heat inactivated FCS. For cytospins, incubation steps 3 and 4 were repeated once, each for 10 min. Between two incubation steps, slides were rinsed with TBS, 0.1% Tween 20, pH 7.5. Slides were then stained for 15 min with the fuchsin substrate-chromogen system (DakoCytomation, K0624). After washing with H_2O, slides were counterstained for 30 sec with Mayer's hematoxylin (Merck, Darmstadt, Germany). After washing again with H_2O, slides were covered with Aquamount (BDH Laboratory Supplies, Poole, England). Microscopical evaluation based on estimating the percentage of cells with strong, medium, weak or no staining. Cells with strong or medium staining were considered positive.

2.2.15. Statistics and general remarks

Assays consisted of duplicate or triplicate values, and at least three independent experiments were performed. Mean values and standard deviations were calculated from these three independent experiments.

The research committee of the Charité-Universitätsmedizin Berlin has approved the described studies. The study was conducted according to the Declaration of Helsinki.