3. Methods

3.1. Centrifugation

If not mentioned otherwise, cells were pelleted by centrifugation at 375 x *g* at room temperature for 4 min. This corresponds to 3125 rpm in an Eppendorf table centrifuge and 1500 rpm in a Varifuge 3.0R (Heraeus).

3.2. Cell culture

The human TE671 rhabdomyosarcoma cell line was obtained from the German collection of microorganisms and cells (DSMZ). Cells were maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine and 10% fetal calf serum (GibcoBRL, Invitrogen), further depicted as complete medium and incubated at 37 °C in 95% air and 5% CO_2. Prior to use, cells were gently trypsinised from the culture flask, washed once in complete medium and resuspended in complete medium. Cells were counted using a Casy1 cell counter (Schärfe System). For co-culture experiments, cells were irradiated with 80 Gy (8000 rad) from a ^{137}Cs source.

3.3. Generation of transfectants

Standard molecular biological methods were performed according to Maniatis, Sambrook & Fritsch (1989) or the manufacturer‘s protocol.

To achieve ectopic and stable expression of CD80 or 4-1BBL, a cDNA library was generated from activated peripheral blood mononuclear cells (PBMC). Next, the full-length cDNA sequences of *CD80* or *4-1BBL* were amplified by primers incorporating a BamHI site at the 5’ end for *CD80* or an EcoRI site at the 3’ end for *4-1BBL*. Subsequently, the PCR-amplified *CD80* or *4-1BBL* fragments were transferred into the vector pGEM-T (Promega) via TA-cloning. The resulting plasmids pGEM-T/*CD80* or pGEM-T/*4-1BBL* and correct sequences were confirmed by DNA cycle sequencing. Then, the *CD80* fragment was recovered by cutting the plasmid with BamHI and NotI and cloned into the eucaryotic expression vector pcDNA3.1+ (Invitro-

gen) at the corresponding positions of the multiple cloning site. Similar, the *4-1BBL* fragment was recovered by cutting the corresponding plasmid with NotI and EcoRI and cloned into pcDNA3.1-.

The resulting plasmids pcDNA3.1+/*CD80*, pcDNA3.1-/*4-1BBL* and, as a mock-control, the empty plasmid pcDNA3.1+ were used to transfect TE671 cells by means of electroporation (Gene Pulser II, Bio-Rad). Stably transfected cells were selected for G418 resistance (0.5 mg/ml) and then subcloned. To assess transgene expression, cells were stained with a PE-labelled mouse anti-human CD80 antibody or mouse anti-human 4-1BBL. Surface expression of the transgenes was determined by flow cytometry on a FACScan (Becton Dickinson).

3.4. T cell culture and activation

3.4.1. Ficoll separation

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors by ficoll separation (Biochrom): One buffy coat was diluted with PBS to give a final volume of 120 ml. 15 ml of ficoll were placed in 50 ml centrifuge tubes and 30 ml of diluted buffy coat were layered on top. The two phase system was centrifuged with 625 x *g* for 25 minutes at room temperature.

Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes, which have been aggregated by the ficoll and, therefore, sediment completely through the ficoll. The layer immediately above the erythrocytes contains mostly granulocytes which at the osmotic pressure of the Ficoll solution attain a density great enough to migrate through the Ficoll layer. Because of their lower density, lymphocytes accumulate at the interface between the plasma and the ficoll.

The lymphocytes were then aspirated from the interface and washed two times in PBS to remove platelets, ficoll and plasma.

3.4.2. T cell enrichment

T cells were enriched by negative selection through incubation with antibodies to CD11b, CD14, CD16 and CD19 followed by magnetic beads conjugated to a secondary anti-mouse IgG antibody (Dynall) according to the manufacturer's protocol. Briefly, PBMCs were suspended in PBS with 0.1% BSA (incubation buffer) at a density of

5 x 10^7/ml and 5 µg of each antibody was added per ml. Cells were incubated for 30 minutes at 4 °C with gentle rotation and washed once with incubation buffer. Then, the cells were resuspended at a density of 2 x 10^7/ml and incubated for another 30 minutes with 100 µl Dynabeads® per ml. Beads with bound cells were then separated with a magnet and the supernatant which contains the T cells was transferred to a new tube and washed once in c-RPMI prior to use. T cell purity was routinely assessed by flow cytometry (see section 3.9., page 39) for CD3 expression and was above 95%.

3.4.3. T cell stimulation

For primary stimulation, cell culture flasks were coated with antibodies to CD3 and CD28, diluted in PBS at a concentration of 2 µg/ml for one hour at 37°C. T cells were stimulated at a final concentration of 1 x 10^6/ml in the presence of plastic bound anti-CD3 and anti-CD28 antibodies for 18 hours. Cells were then pelleted and resuspended in complete medium containing 100 U/ml recombinant IL-2 (Chiron) and transferred to a new flask. At day 5, dead cells were removed by ficoll separation. Vital T cells were resuspended in complete medium without IL-2 and restimulated in the presence or absence of transduced TE671 cells. T lymphocytes were employed at a final concentration of 5 x 10^5/ml and transfectants were added at a final density of 1 x 10^5/ml (E:T = 5:1). For restimulation, OKT3 was used at a concentration of 1 µg/ml and cross-linked by the use of $F(ab')_2$ fragment goat anti-mouse IgG at 1.3 µg/ml. As a control, an isotype matched control antibody was used instead of OKT3. In inhibition experiments, T cells were preincubated with inhibitors or DMSO one hour prior to restimulation.

3.5. Quantification of apoptotic and necrotic cell death

3.5.1. Single cell measurement of genomic DNA fragmentation

After treatment, 1 x 10^5 T cells were pelleted in a 96-well U-bottom plate. The pellet was then gently resuspended with 200 µl Nicoletti-buffer (50 µg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) and incubated overnight in the dark at 4 °C. Genomic DNA content was determined using a FACScan flow cytometer (Becton Dickinson) and CellQuest analysis software. Nuclei displaying a hypodiploid, sub-G_1 DNA content were identified as apoptotic (Nicoletti et al. 1991). Cell debris, characterised by low forward and side scatter values, was excluded from the analysis.

3.5.2. Annexin-V-FITC

To distinguish apoptotic from necrotic cell death, tumour cells were stained with Annexin-V-FITC and counterstained with propidium iodide (PI). Annexin-V-FITC binds to phosphatidylserine (PS) on the outer leaflet of the plasma membrane. PI is excluded by cells with intact membranes. PI positivity is therefore a sign of necrosis, whereas cells positive for Annexin-V, but negative for PI are generally defined as apoptotic (Vermes et al. 1995, 1997).
Cells were washed twice with cold PBS and resuspended in Annexin binding buffer (10 mM N-(2-hydroxyethyl)piperacine-N‘-3(propansulfonicacid)/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at 1 x 10^6 cells/ml. Next, 2 µl of Annexin-V-FITC (BD PharMingen) and 4 µl PI (20 µg/ml, Sigma-Aldrich) were added to 100 µl cell suspension. Samples were incubated for 20 minutes in the dark at room temperature. After incubation, 100 µl Annexin binding buffer was added. Analyses were performed using a FACScan (Becton Dickinson) and CellQuest analysis software.

3.6. Measurement of T cell proliferation

24 hours after restimulation, T cells were transferred into flat-bottom 96-well plates and 1 µCi ^{3}H-thymidine (#TRA61, Amersham) was added per well for 18 hours. Cells were then harvested onto glass fiber filter plates (PerkinElmer) and ^{3}H-thymidine incorporation was quantified by liquid scintillation counting (Top Count, PerkinElmer). This assay was performed at least in triplicate.

3.7. Measurement of T cell cytotoxicity

For detection of cytotoxic T cell function, non-irradiated or irradiated tumour cells (TE.mock or TE.CD80) were labelled at a density of 6 x 10^6/ml with 1 mCi/ml ^{51}Cr (NEN) in c-RPMI for 1.5 hours. The labelled tumour cells were then washed three times with D-PBS and added to the pre-activated T-effector cells at effector-target ratios (E:T) ranging from 100:1 to 1.2:1. As internal controls, spontaneous and maximal lysis were determined by adding medium or lysis buffer (see section 3.8., page 37) instead of T cells, respectively. After 4 hours of co-culture in 96-well V-bottom plates, cytotoxic T cell activity was assessed by measuring ^{51}Cr in culture supernatants by

scintillation counting (Top Count, PerkinElmer). Specific lysis was calculated as [(sample cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] x 100%. This assay was performed in triplicate.

3.8. Immunoblotting

3.8.1. Sample preparation

After restimulation, T cells were washed twice with PBS, and lysisbuffer was added to the pellet. 100 µl lysisbuffer were used for a 40 ml culture, containing approximately 2 x 10^7 T cells. Samples were vortexed thoroughly and kept on ice for 15 minutes. Cell debris was spun down at 16000 x *g* and the supernatant was collected as sample.

Lysisbuffer:	2 mM EDTA 0.1% SDS 1% Triton-X100 1 mM Na_3VO_4 1 mM β-glycerolphosphate Protease inhibitor cocktail ("Complete", Roche Diagnostics)

3.8.2. Determination of protein concentration

Protein concentration was determined using the bicinchoninic acid assay (Smith et al. 1985) from Pierce according to the manufacturer's protocol. Briefly, 5 µl of sample were prediluted 1:5 in lysis buffer and to 10 µl of the prediluted sample 200 µl of BCA solution were added in an ELISA plate (flat bottom) and incubated at 37 °C for 30 minutes in the dark. Absorption was measured using an ELISA reader at 590 nm. BSA was used as standard and the protein concentration was calculated on the basis of the derived standard curve. The assay was performed in duplicate.
Protein concentration was equalised by diluting with lysis buffer. Then, 5 x sample buffer was added and the samples were boiled for 5 minutes.

5 x Sample buffer:	260 mM Tris/Cl, pH 6.8 400 mM Dithiothreitol (DTT) 40% Glycerol 0.004% Bromphenolblue 2% SDS

3.8.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In SDS-PAGE, migration of proteins is determined not by intrinsic electric charge but by molecular weight. SDS, an anionic detergent, denaturates proteins by wrapping the hydrophobic tail around the polypeptide backbone, giving the protein a negative charge proportional to its length. The reducing agent dithiothreitol in the sample buffer cleaves any disulfide bonds between cysteine residues, resulting in a completely unfolded protein. To achieve high resolution, a discontinuous system was used (Laemmli et al. 1970). Here, proteins are concentrated in a stacking gel before entering the separating gel. Electrophoresis was carried out with a Bio-Rad Mini-PROTEAN® 3 system. Gels were prepared with 0.75 mm spacers and 10 or 15 pockets. The solutions indicated in the table are sufficient for 2 mini gels. Equal amount of protein (15 µg per lane) was loaded on the gel and electrophoresis was performed at 120 V for the first 5 minutes and continued with 200 V until the bromphenolblue front left the gel.

Separating gel (16%):	3.3 ml Aqua dest. 4 ml Acrylamide/Bisacrylamide solution (29:1; 40%) 2.5 ml 1.5M Tris/Cl, pH 8.8 100 µl 10% SDS 100 µl 10% APS 4 µl TEMED
Stacking gel (5%):	2.185 ml Aqua dest. 375 µl Acrylamide/Bisacrylamide solution (29:1; 40%) 380 µl 0.5M Tris/Cl, pH 6.8 30 µl 10% SDS 30 µl 10% APS 3 µl TEMED
10 x Running buffer	250 mM Tris 1% SDS 2 M Tris

3.8.4. Electroblotting

For Western blot analysis, electrophoretically separated proteins were transferred onto 0.2 µm nitrocellulose membranes (Schleicher & Schuell) by semi-dry blotting using a Bio-Rad Trans-blot SD transfer cell. Membranes and filter paper (Schleicher & Schuell GB005) were incubated in CAPS buffer for several minutes. A blot sandwich was made out of anode, 2 layers filter paper, membrane, gel, 2 layers filter paper and cathode. Membrane and paper fitted the gel‘s size and blotting was performed at

1 mA/cm^2 for one hour. Membranes were then stained with ponceau red to verify homogenous transfer and destained in aqua dest.. Dried membranes were stored in paper bags at room temperature.

CAPS buffer:	10 mM CAPS, pH 11 10% Methanol
Ponceau red:	0.1% Ponceau-S in 5% acetic acid

3.8.5. Immunodetection of proteins

The membranes with the transferred proteins were then preblocked for one hour in blocking buffer and incubated for one hour with primary antibody, diluted in blocking buffer. After washing three times with blocking buffer, the membranes were incubated for one hour with horseradish peroxidase-conjugated secondary antibody, diluted in blocking buffer. Finally, the membranes were washed three times with PBST and developed using the enhanced chemiluminescence system (Amersham Biosciences). The developed films were scanned by use of a GS700 imaging densitomer (Bio-Rad, Hercules, CA) and band densities, expressed as "OD x mm", were assessed with the densitomer's software. Optical density (OD) is expressed as a relative unit in relation to the height of the band as measured in mm.

Blocking buffer	10% Casein (Roche) 0.1% Tween in PBS
PBST	0.1% Tween in PBS

3.9. Immunocytometry

To quantify expression of surface antigens, 2 x 10^5 cells were washed with PBS containing 10% FCS (staining buffer) and resuspended in 100 µl staining buffer. The samples were then incubated with 2 µl PE-labelled antibody or the corresponding control antibody for 30' on ice. Then, the cells were washed two times with staining buffer

and analysed using a FACScan flow cytometer (Becton Dickinson) and CellQuest analysis software. Dead cells and debris were excluded from the analysis by setting an appropriate gate.

3.10. Immunocytochemistry

Morphology TE671 cells was assessed by light microscopy. Cells were panoptically stained or immunophenotyped. Briefly, 3 x 10^4 TE671 cells, previously irradiated or treated with freeze and thaw cycles, were spun down onto glass-slides using a Shandon Cytospin 3 centrifuge (Thermo Shandon). After drying, the cells were stained with May-Grünwald-Giemsa stain and assessed with an Olympus BX50 microscope at 100 x magnification. Alternatively, 5 x 10^4 cells were incubated in 50 µl PBS containing 10% FCS (staining buffer) and 10 µg/ml monoclonal mouse anti-human CD80 antibody (Immunotech) for 30 minutes at 4 °C. Cells were washed twice with staining buffer and stained with 10 µg/ml Alexa Fluor 594-conjugated chicken anti-mouse IgG (Molecular Probes) diluted in 50 µl staining buffer for 30 minutes at 4°C. Then, cells were washed twice and resuspended in 150 µl staining buffer. Cells were centrifuged onto glass-slides using a Shandon Cytospin 3 centrifuge. After drying, the cells were mounted in Dako fluorescent mounting medium (DakoCytomation) and inspected.