4. Results

4.1. Purification of primary human T cells

To study costimulatory signals on T cells in my model system, T lymphocytes had to be separated from the rest of the lymphocytes. There are two ways of obtaining purified T cells from a mixture of PBMCs: Positive and negative selection, i.e. depletion. In the first method, T cells are selected by specific antibodies to the T cell markers CD3 or CD4 and CD8. As positive selection bears the risk of an uncontrolled T cell activation, I decided to perform a negative selection instead. This was achieved by adding antibodies directed against CD11b, CD14, CD16 and CD19. These molecules are expressed on the surface of monocytes, granulocytes, macrophages, NK cells and B cells. Subsequently cells that had been targeted with one of these antibodies were removed by adding a secondary antibody which was coupled to a magnetic bead.

The T cell purity achieved by this method was above 95%. Fig. 4 illustrates the T cell enrichment of a peripheral blood sample.
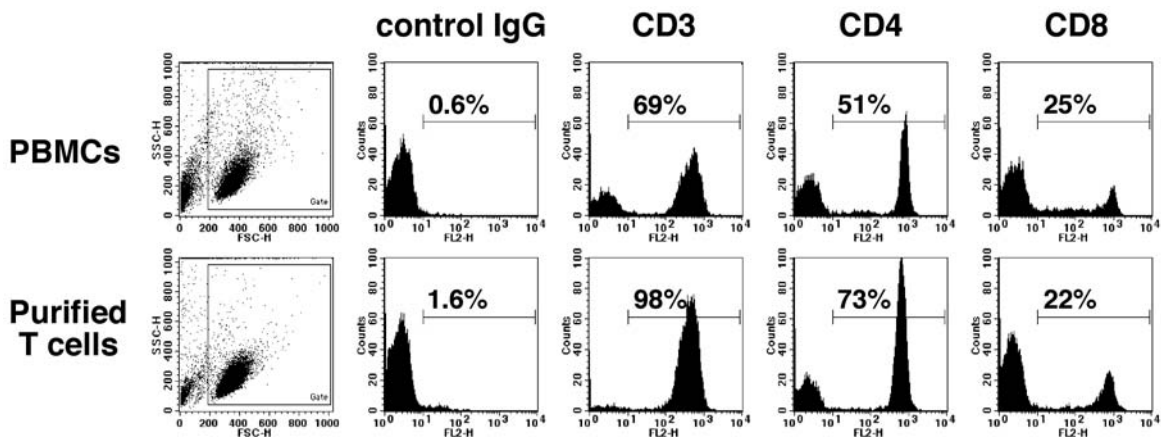

Fig. 4: Purification of T cells. The histograms show the expression levels of T cell antigens on PBMCs before and after purification. Cells were stained with the indicated PE-labelled antibodies and analysed by flow cytometry. The dot plots (first column) show the gate that was set to exclude dead cells and debris from the analysis.

4.2. Culture and stimulation of primary human T cells

To characterise my system, purified T cells were activated through ligation of CD3 and CD28 on T cells and surface expression of CD4, CD8 and CD95 was quantified at various timepoints using FACS analysis (Fig. 5A). Moreover, absolute cell numbers of these T cell subsets were monitored at distinct time points (Fig. 5B).
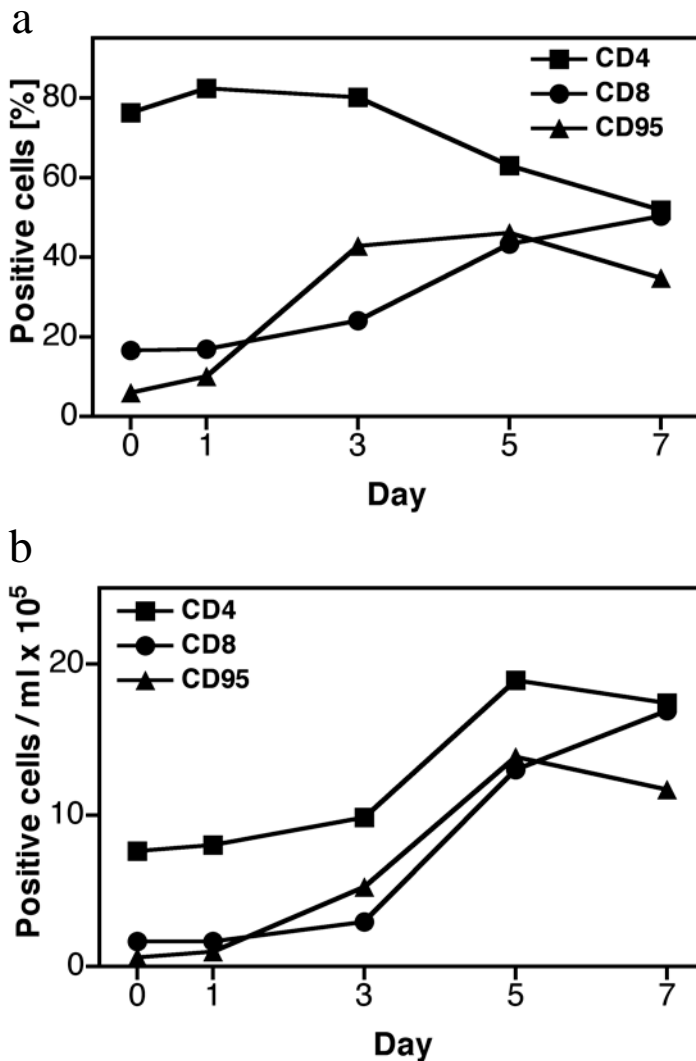

Fig. 5: Activated T cells in prolonged culture. Purified T cells were stimulated with plastic bound antibodies to CD3 and CD28 for 18 hours and then cultured in medium containing 100 U/ml recombinant IL-2. (A). Expression of CD4, CD8 and CD95 was determined at the indicated timepoints by FACS analysis. (B). Total cell concentration was determined at the indicated timepoints and correlated with the percentage of positive cells.

4.3. 4-1BB ligand costimulates proliferation of activated T cells and inhibits AICD

Signals mediated through costimulatory molecules facilitate T cell proliferation during restimulation. To analyse their role during the inhibition of AICD, I employed the rhabdomyosarcoma cell line TE671 to generate transfectants stably expressing the ligands of the costimulatory molecules 4-1BB or CD28, i.e. 4-1BB ligand (TE.4-1BBL) or CD80 (TE.CD80), respectively (Fig. 6). Additionally, transfectants solely containing the vector without a transgene (TE.mock) were generated. None of the cell lines expressed the costimulatory ligands CD86 or OX40 ligand (data not shown). To test the costimulatory capacity of these transfectants, purified T cells, previously activated through CD3 and CD28 ligation, were co-cultured with TE.mock in the presence of the anti-CD3 antibody OKT3 or the IgG isotype control. There, proliferation amounted to 14035 cpm (SD ±2563) and 13418 cpm (SD ±3180), respectively (Fig. 7A). In contrast, use of TE.CD80 or TE.4-1BBL enhanced T cell proliferation upon CD3 ligation but not in the presence of the IgG control antibody (Fig. 7A).

Subsequently, transfectants were tested for their capacity to inhibit AICD. To this end, T cells, primarily activated with plastic bound anti-CD3 and anti-CD28 antibodies, were restimulated on day five after primary activation through co-culture with differ-
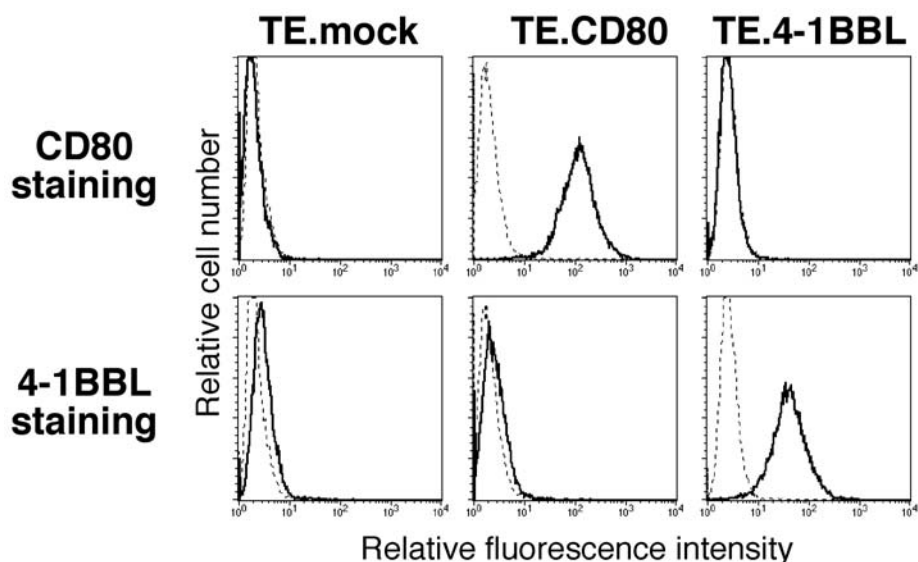

Fig. 6: Expression of costimulatory molecules on stably transfected tumour cells. Transgene expression was measured by FACS analysis using PE-labelled mouse anti-human CD80 or 4-1BBL antibodies (solid lines). As a control, a PE-labelled isotype matched antibody was used (dashed line).

ent transfectants in the presence of anti-CD3 or the control antibody. Co-culture of T cells with either TE.CD80 or TE.4-1BBL in the presence of anti-CD3 antibody resulted in a decrease of AICD (Fig. 7B).
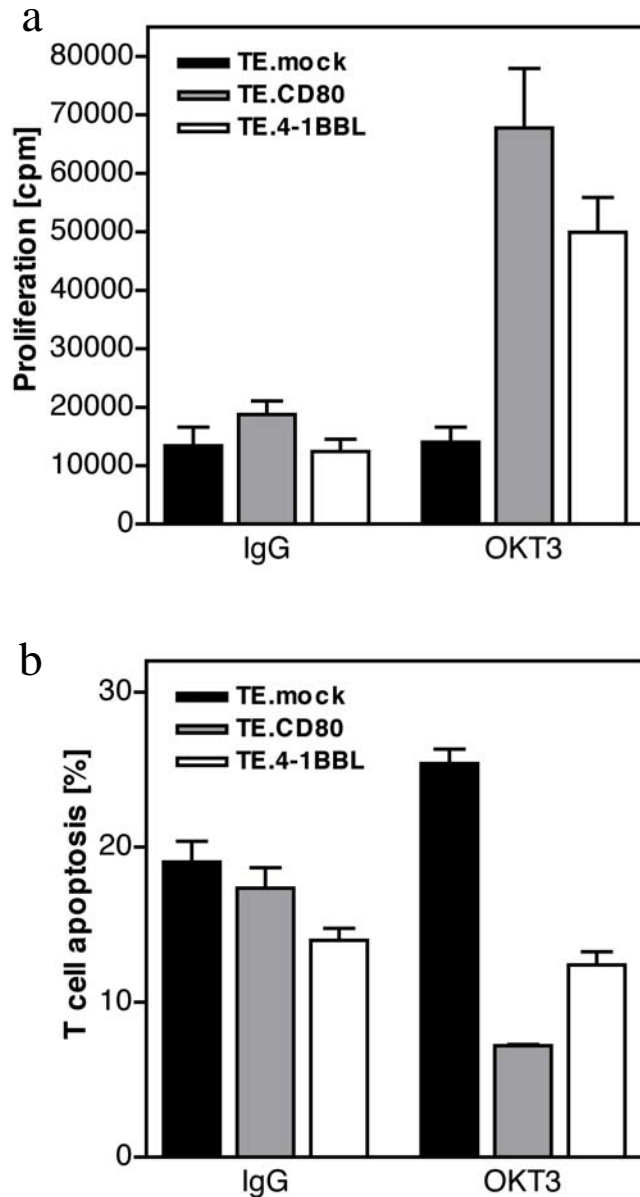

Fig. 7: Costimulation induces T cell proliferation and inhibition of AICD. (A). Induction of T lymphocyte proliferation. Pre-activated T cells were restimulated with cross-linked anti-CD3 antibodies (OKT3) in the presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As a control, an isotype-matched IgG was used. After 24 hours, T cells were transferred into a fresh 96-well plate and pulsed for 18 hours with 1 µCi ^{3}H-thymidine per well. Proliferation was determined as ^{3}H-thymidine incorporation. Mean values and standard deviations of an experiment performed in quadruplicate are shown. The experiments are representative of three independent experiments performed from different blood donors.

(B). Inhibition of activation-induced cell death. Pre-activated T cells were restimulated with cross-linked anti-CD3 antibodies (OKT3) in the presence of mock-, CD80- or 4-1BBL-transfected TE671 cells (TE.CD80 or TE.4-1BB). As a control, an isotype-matched IgG antibody was used. After 24 hours of co-culture, T cells were transferred into a fresh 96-well plate in order to separate T lymphocytes from stimulator cells. T cells were then cultured another 24 hours and apoptosis was quantified as described under materials and methods. Mean values and standard deviations of an experiment performed in triplicate are shown. The experiments are representative of three independent experiments performed from different blood donors.

4.4. Bcl-x$_L$ and c-FLIP$_S$ are up-regulated upon costimulation through 4-1BB

Published data demonstrated that T cell restimulation through CD3 along with an agonistic anti-CD28 antibody results in up-regulation of Bcl-x$_L$ (Boise et al. 1995) and c-FLIP$_S$ (Kirchhoff et al. 2000), thus blocking both the intrinsic and the extrinsic pathway of apoptosis. In line with this, I could show in my system that reactivation of T cells with anti-CD3 antibodies in the presence of CD80 expressing allogeneous tumour cells induced up-regulation of Bcl-x$_L$ (Fig. 8A) and c-FLIP$_S$ (Fig. 8B). Furthermore, when analysing the molecular effects of 4-1BB-mediated costimulation, I ob-
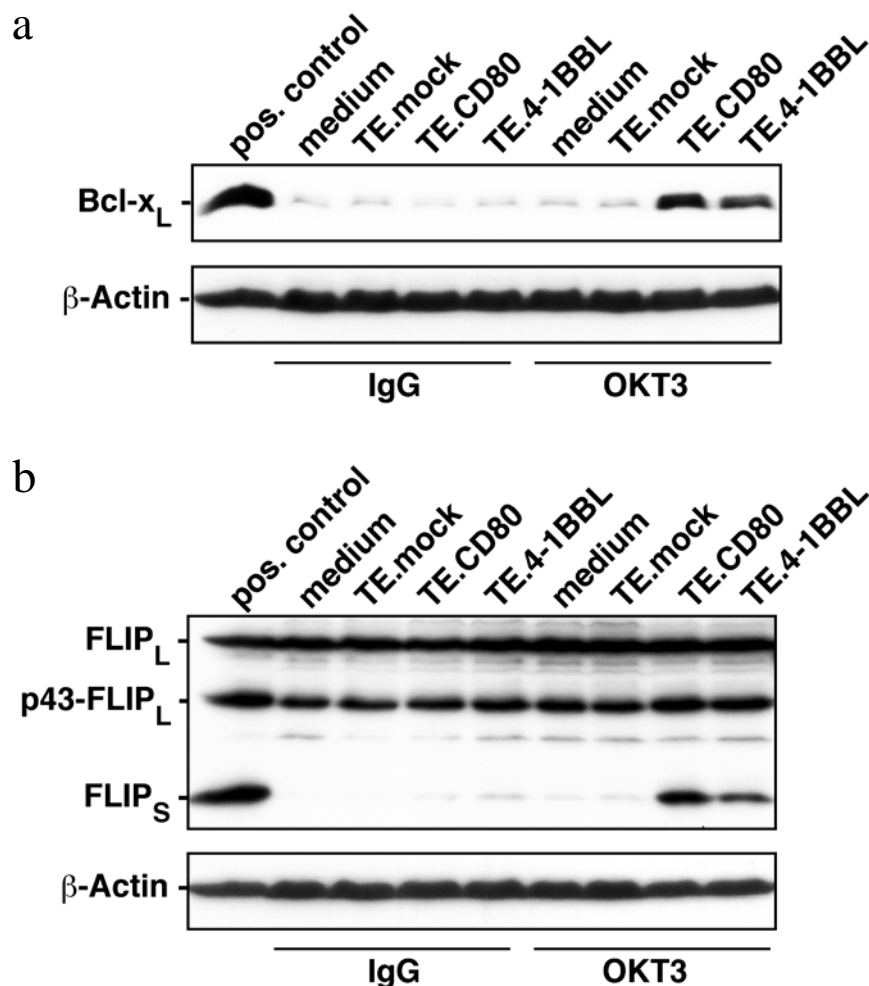

Fig. 8: Costimulation mediates up-regulation of Bcl-x$_L$ and FLIP$_S$. Pre-activated T cells were restimulated with cross-linked anti-CD3 antibodies (OKT3) in the absence (medium) or presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As a control, an isotype-matched IgG was used. After 24 hours, western blot analysis was performed for Bcl-x$_L$ (A) and FLIP$_S$ (B). Equal protein loading was confirmed by immunoblotting using an anti-actin antibody.

served a similar up-regulation of Bcl-x$_L$ (Fig. 8A) and of c-FLIP$_S$ (Fig. 8B) in T lymphocytes that were stimulated through CD3 and 4-1BB as compared to T cells stimulated through CD3 alone. Interestingly, while kinetics of Bcl-x$_L$ and c-FLIP$_S$ expression demonstrated peak levels at 6 or 24 hours, a slight up-regulation of c-FLIP$_S$ was also visible at 6 hours if pre-activated T cells were co-cultured with TE.mock cells in the presence of anti-CD3 antibody (Fig. 9).
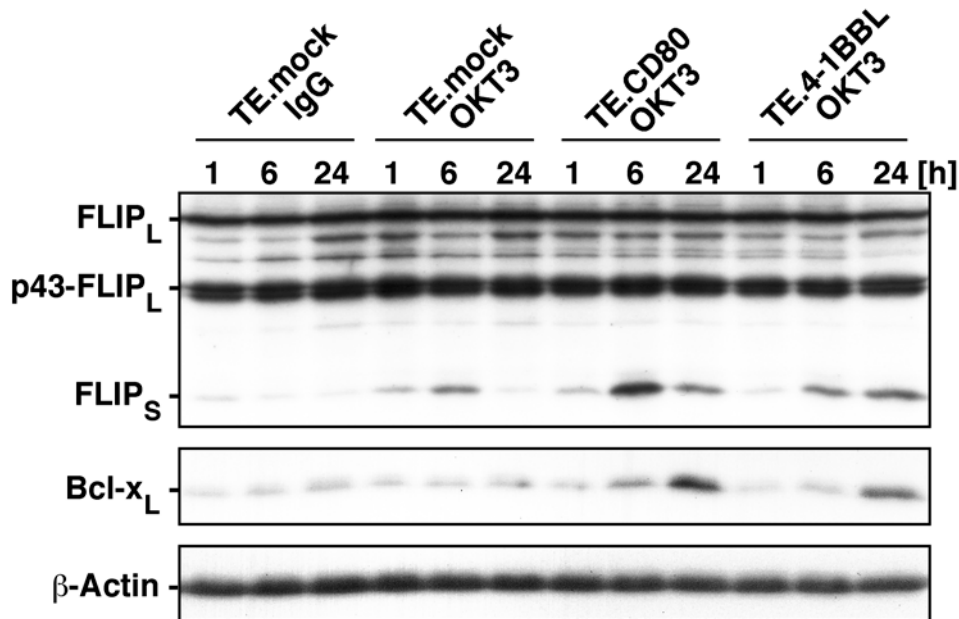

Fig. 9: Kinetics of Bcl-x$_L$ and FLIP$_S$ up-regulation. Pre-activated T cells were restimulated for the indicated periods with cross-linked anti-CD3 antibodies (OKT3) in the presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As a control, T cells co-cultured with TE.mock and an isotype-matched IgG were assessed. Western blot analysis for FLIP$_S$ and Bcl-x$_L$ are shown. Equal protein loading was confirmed by immunoblotting using an anti-actin antibody.

4.5. Inhibition of PI3 kinase interferes with 4-1BB-mediated inhibition of AICD and hampers Bcl-x$_L$ and c-FLIP$_S$ up-regulation

CD28-mediated signals have been shown to result in PI3 kinase (Prasad et al. 1994) and AKT (Kane et al. 2001) activation. Furthermore, CD28 ligation and AKT phosphorylation have been implied in c-FLIP$_S$ regulation (Kirchhoff et al. 2000). In contrast, 4-1BB, to date, has been shown to recruit TRAF-1 and -2 (Arch & Thompson 1998) and subsequently activates JNK/SAPK, p38 MAPK (Cannons et al. 1999) and NF-κB (Arch & Thompson 1998).

To investigate the importance of PI3 kinase for 4-1BB-mediated apoptosis inhibition, I tested whether the blockade of PI3 kinase interferes with costimulation-mediated T cell effector functions. To this end, pre-activated T cells were treated with increasing amounts of the PI3 kinase inhibitor LY 294002 for one hour prior to restimulation. Subsequently, T cells were restimulated through CD3 ligation and cultured in the presence of TE.mock, TE.CD80 or TE.4-1BBL cells. As controls, pre-activated T lymphocytes were cultured in media containing the IgG control antibody or 0.05% DMSO as a solvent control. T lymphocyte proliferation, as induced by TCR/CD3 stimulation and co-culture with TE.CD80 cells, decreased from 80343 cpm (SD ±3583) in the absence to 6651 cpm (SD ±970) in the presence of LY 294002 (20 µM final concentration) (Fig. 10A). Furthermore, AICD increased from 7.2% (SD ±0.07) to 29.5% (SD ±0.69) when LY 294002 was added (Fig. 10B). Interestingly, similar results were observed when activated T lymphocytes co-cultured with TE.4-1BBL cells were exposed to LY 294002. There, proliferation dropped from 62128 cpm (SD ±5731) in the absence of the inhibitor to 6914 cpm (SD ±492) in the presence of LY 294002 (Fig. 10A). Likewise, AICD increased from 12.4% (SD ±0.84) to 29.8% (SD ±0.55) when LY 294002 was added (Fig. 10B).

It is of note that pre-activated T lymphocytes upon co-culture with the 4-1BBL expressing transfectants, in the absence of a CD3-mediated signal, demonstrated a moderate increase of proliferation and a fair decrease of apoptosis as compared to T cells that were cultured with mock transfectants alone (Fig. 10). A similar, albeit smaller, increase of proliferation was observed when pre-activated T cells were cultured with CD80 expressing transfectants alone.

To examine the effects of the PI3 kinase blockade on downstream signals, AKT phosphorylation was determined. Here, activated T cells were treated for one hour with IgG or anti-CD3 in the presence of TE.mock, TE.CD80 or TE.4-1BBL cells. When assessing AKT phosphorylation, I observed that culture of activated T cells with anti-CD3 antibody and TE.mock cells facilitated AKT phosphorylation even in the absence of a costimulatory signal. However, AKT phosphorylation slightly increased if T cells were costimulated through CD28 or 4-1BB (Fig. 11). Accordingly, optical density (OD) of phosphorylated AKT depicted in figure 11 increased from 106 OD x mm when T cells were stimulated with anti-CD3 in the presence of TE.mock to 156 OD x mm or 152 OD x mm when T cells were triggered with anti-CD3 in the presence of
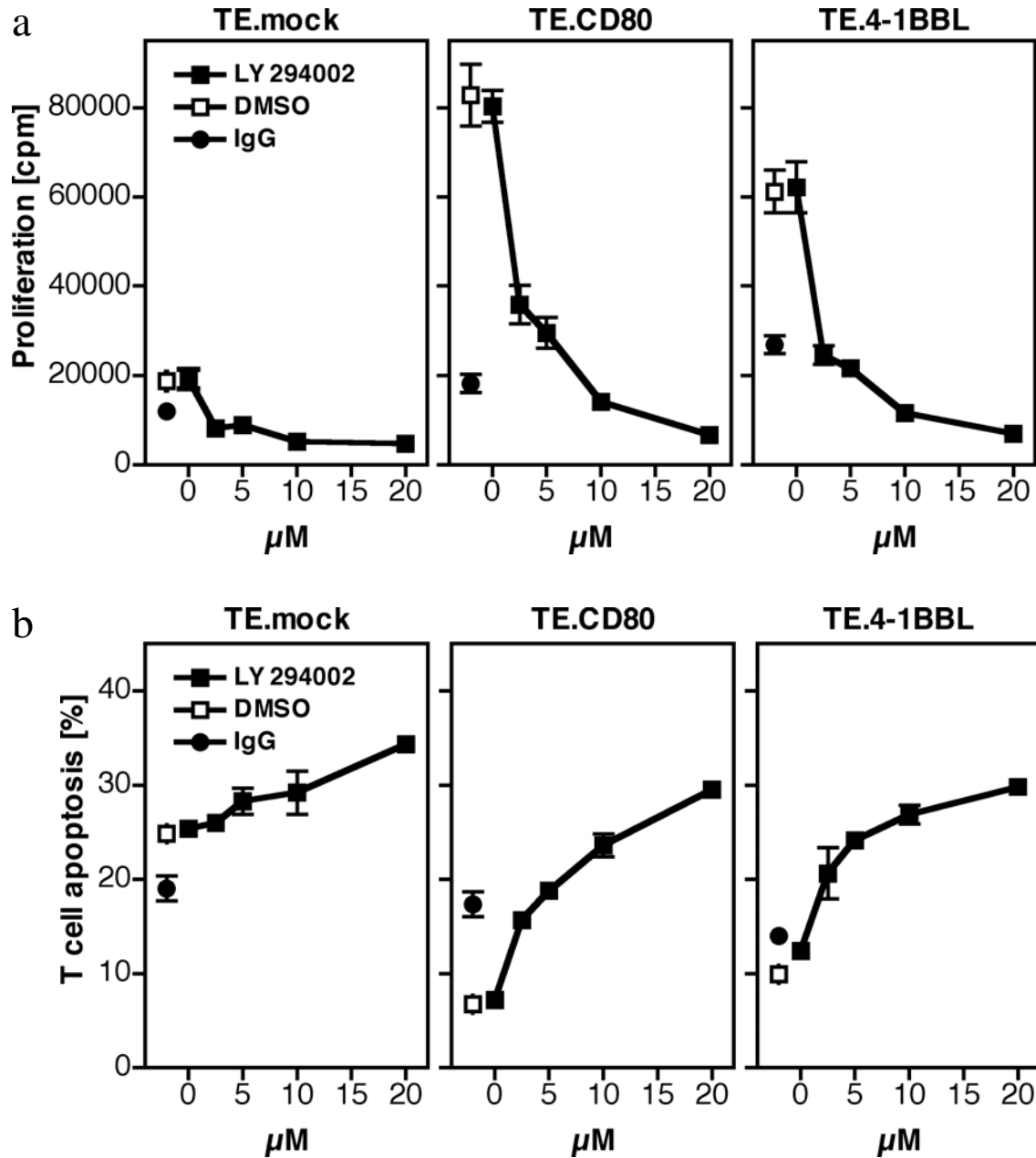
Fig. 10: T cell proliferation and AICD inhibition are PI3 kinase-dependent. *Pre-activated T cells were treated with increasing amounts of the PI3 kinase inhibitor LY 294002, as indicated, and restimulated with cross-linked anti-CD3 antibodies (OKT3) in the presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As a control, restimulated T lymphocytes were either cultured with DMSO containing medium (0.05% final concentration) or in the presence of an isotype-matched IgG control. After 24 hours of co-culture, T cells were transferred into a fresh 96-well plate. (A). Subsequently, T cells were pulsed for additional 18 hours with 1 µCi ^{3}H-thymidine per well and proliferation was determined as ^{3}H-thymidine incorporation. (B). Alternatively, T cells were cultured for an additional 24 hours and apoptosis was assessed as described under material and methods. Mean values and standard deviations from an experiment performed in triplicate are shown.*

TE.CD80 or TE.4-1BBL, respectively. Thus, for AKT phosphorylation, signals mediated through TCR/CD3 appear to synergise with signals induced by CD28 or 4-1BB. Finally, when testing whether the inhibition of PI3 kinase activity blocked AKT phosphorylation in my system, LY 294002 nearly completely abolished AKT phosphorylation in all conditions assessed (Fig. 11).
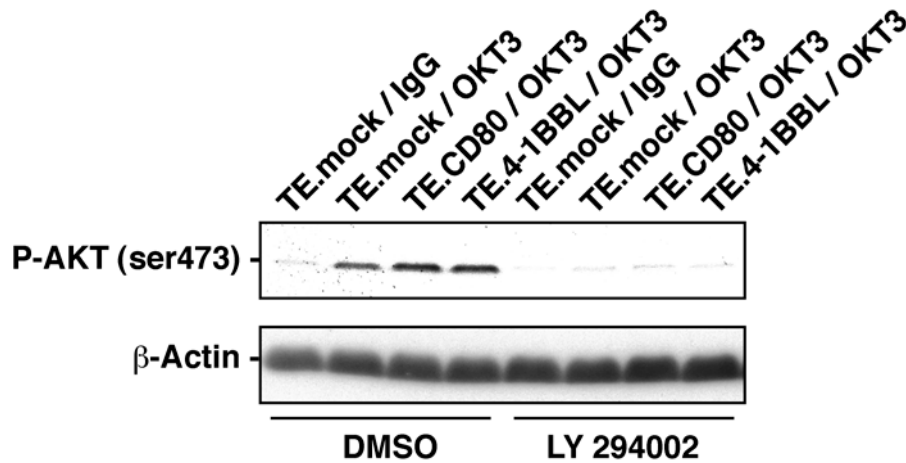

Fig. 11: AKT phosphorylation upon T cell restimulation is PI3 kinase-dependent. Pre-activated T cells were restimulated for one hour with cross-linked anti-CD3 antibodies (OKT3) in the presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As a control, T cells co-cultured with TE.mock and an isotype-matched IgG were assessed. Cells were either treated with DMSO (0.05% final concentration) or cultured with the PI3 kinase inhibitor LY 294002 (10 μM final concentration). Western blot analysis for phospho-AKT (ser473) are depicted. Equal protein loading was confirmed by immunoblotting using an anti-actin antibody.

To further corroborate the importance of PI3 kinase activation during AICD inhibition, I subsequently tested whether PI3 kinase inhibition influenced the expression of the anti-apoptotic molecules Bcl-x_L or c-FLIP$_S$. To this end, pre-activated T cells were restimulated through CD3 and co-cultured with TE.mock, TE.CD80 or TE.4-1BBL cells. When assessing Bcl-x_L and c-FLIP$_S$ expression, up-regulation was detectable if T cells were restimulated through CD3 and co-cultured with TE.CD80 or TE.4-1BBL cells (Fig. 12). However, neither Bcl-x_L (Fig. 12A) nor c-FLIP$_S$ (Fig. 12B) were measurable if cells had been treated with LY 294002.

Thus, inhibition of PI3 kinase, an upstream regulator of AKT, prevented the effects of costimulatory signalling, i.e. proliferation, inhibition of AICD, phosphorylation of AKT and up-regulation of anti-apoptotic proteins.
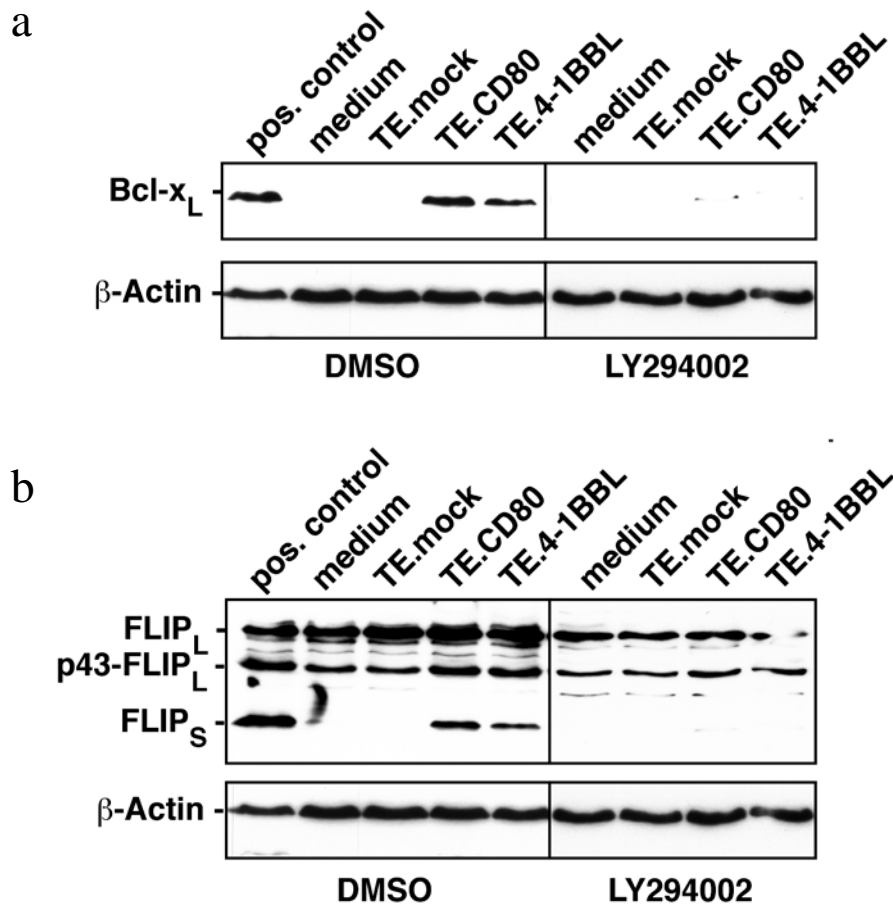
a

Bcl-x$_L$ -

β-Actin -

DMSO   LY294002

b

FLIP$_L$ -
p43-FLIP$_L$ -

FLIP$_S$ -

β-Actin -

DMSO   LY294002

Fig. 12: Costimulation mediated up-regulation of FLIP$_S$ and Bcl-x$_L$ is PI3 kinase-dependent.
Pre-activated T lymphocytes were treated with the PI3 kinase inhibitor LY 294002 (10 µM final concentration) and re-stimulated for 24 hours with cross-linked anti-CD3 antibodies (OKT3) in the absence (medium) or presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As a control, cells were treated with the corresponding dose of DMSO. (A). Western blot analysis for Bcl-x$_L$. (B). Western blot analysis for FLIP$_S$. Equal protein loading was confirmed by immunoblotting using an anti-actin antibody.

4.6. Inhibition of AKT phosphorylation decreases 4-1BB-mediated proliferation and inhibition of apoptosis as well as Bcl-x$_L$ and c-FLIP$_S$ up-regulation

To further elucidate a role for AKT during CD28- or 4-1BB-mediated proliferation and AICD inhibition, T cells were pre-cultured for one hour with different amounts of the AKT inhibitor SH-5. This blocking agent is a modified phosphoinositol ana-
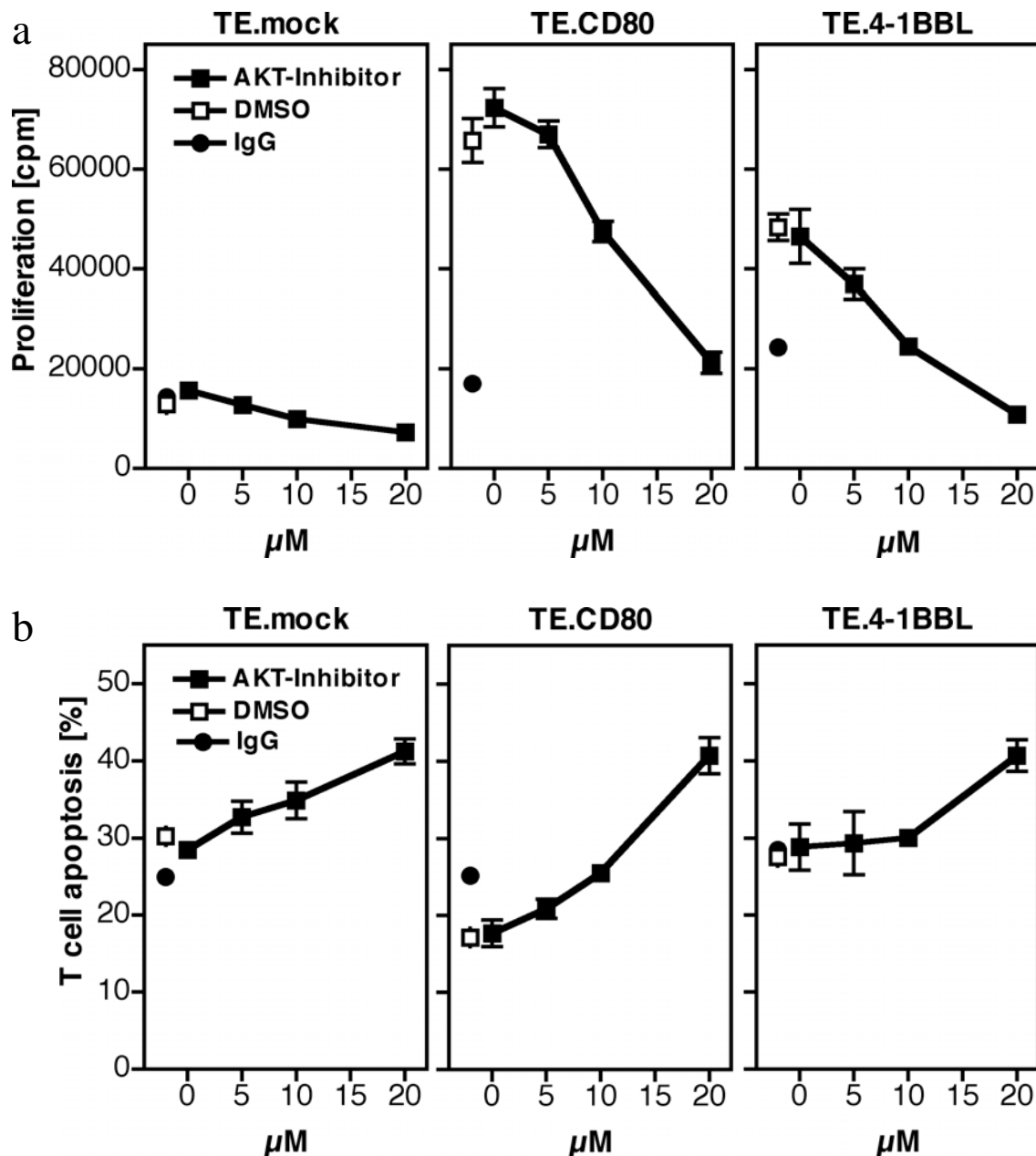

Fig. 13: T lymphocyte proliferation and AICD inhibition are AKT-dependent. Cells were treated as described in figure 3, but instead of the PI3 kinase inhibitor LY 294002 the AKT inhibitor SH-5 was used at increasing amounts, as indicated. Assessment of T lymphocyte proliferation (A) and inhibition of AICD (B) are depicted.

logue that selectively inhibits activation of AKT without affecting activation of PDK1, another PI3 kinase regulated kinase (Kozikowski et al. 2003). Similar to my results with LY 294002, AKT inhibitor SH-5 effectively blocked CD28- or 4-1BB-mediated T cell proliferation (Fig. 13A) and inhibition of AICD (Fig. 13B). Furthermore, 4-1BB-induced up-regulation of the anti-apoptotic effector molecules Bcl-x_L (Fig.
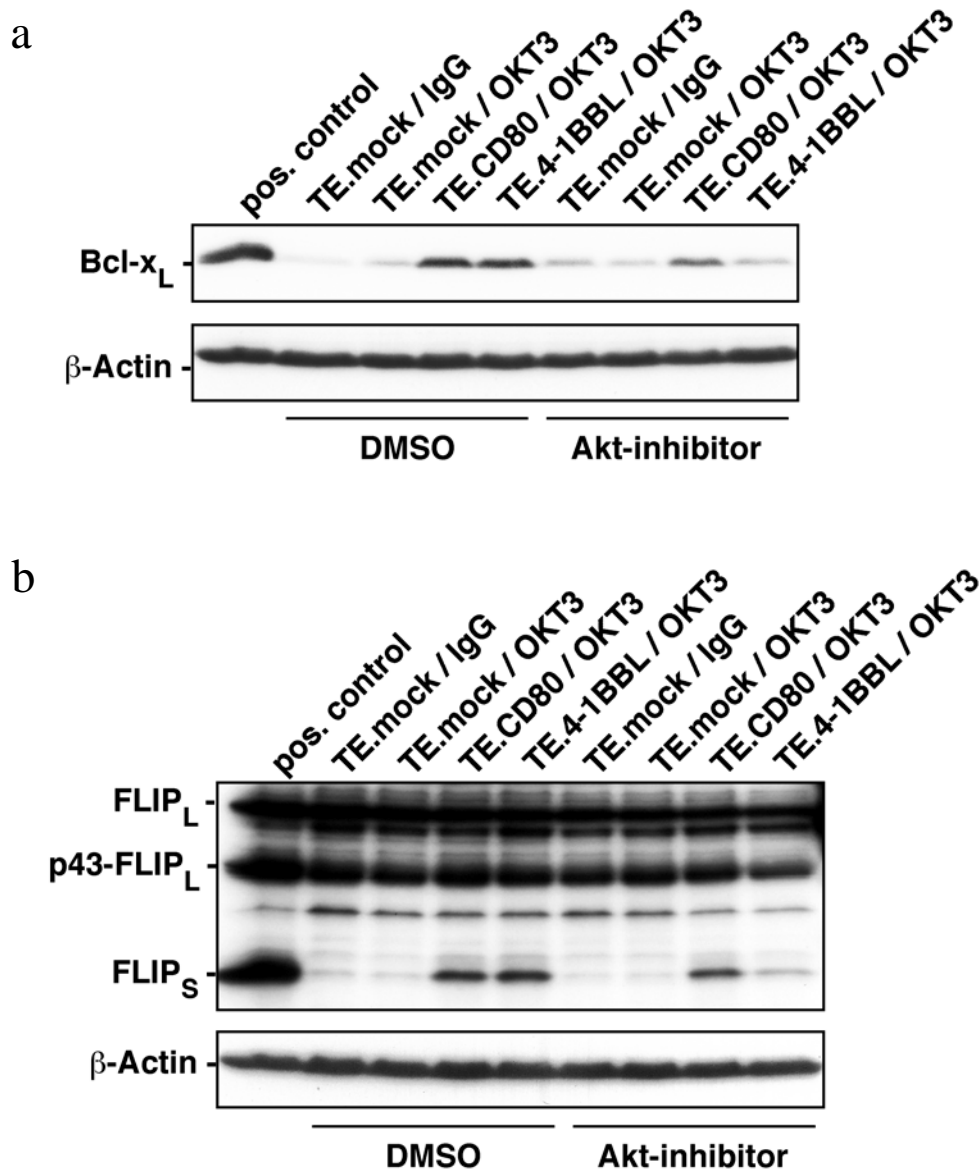

Fig. 14: Costimulation mediated up-regulation of FLIP$_S$ and Bcl-x_L is AKT-dependent. *P r e - a c - tivated T cells were treated with the AKT inhibitor SH-5 (20 μM final concentration) one hour pri- or to restimulation and were restimulated for 24 hours with cross-linked anti-CD3 antibodies (OKT3) in the presence of mock-, CD80- or 4-1BBL-transfected TE671 cells. As controls, cells were co-cultured with TE.mock, the isotype IgG control and DMSO (0.05% final concentration). (A). Western blot analysis for Bcl-x_L. (B). Western blot analysis for FLIP$_S$. Equal protein loading was confirmed by immunoblotting using an anti-actin antibody.*

14A) and c-FLIP$_S$ (Fig. 14B) was largely hampered if the AKT inhibitor SH-5 was employed. This indicates that AKT activation is a prerequisite for 4-1BB-mediated T cell proliferation and AICD inhibition via Bcl-x$_L$ and c-FLIP$_S$.

4.7. Tumour cell irradiation does not influence transgene expression

A variety of reports demonstrated that expression of costimulatory molecules increases the immune modulatory potential of tumour cells. In line with this, tumour cell transfection with CD80 has been shown to facilitate T cell effector functions, for example proliferation and tumour cell lysis and to inhibit activation-induced T cell death (Daniel et al. 1997a). Irradiation of such tumour cell vaccines prior to transfer into patients is a prerequisite in many human preclinical and clinical trials. Previous data indicated that ionising irradiation impairs the generation of tumour immunity *in vivo* (Cayeux et al. 1995, 1996; Hock et al. 1993a). I therefore asked whether tumour cell irradiation diminishes the immune modulatory capacity or affects signalling for T cell survival, i.e. interferes with inhibition of T cell apoptosis by CD80 expressing cells.

First, I determined whether irradiation interferes with transgene expression in the rhabdomyosarcoma cell line TE671. To this end, TE671 tumour cells, stably transfected to express CD80 (TE.CD80), were irradiated with 80 Gy or 200 Gy by the use of a ^{137}Cs source. Subsequently, CD80 expression was measured immediately after, 24 and 48 hours after irradiation and compared to CD80 expression prior to inactivation. Neither irradiation at 80 Gy (Fig. 15) nor at 200 Gy (data not shown) decreased CD80 expression levels.
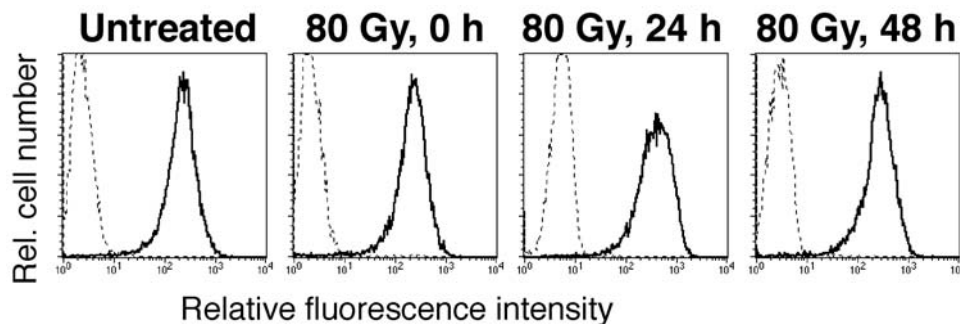

Fig. 15: CD80 expression on stably transfected TE671 cells following irradiation. Transgene expression was measured at the indicated time points following irradiation with 80 Gy. Tumour cells were stained by the use of a PE-labelled mouse anti-human CD80 antibody (solid line) or a PE-labelled isotype-matched control antibody (dashed line). Surface expression of CD80 was determined by flow cytometry measuring ten thousand cells from each sample.

4.8. Irradiation does not affect target cell lysis

Tumour cells expressing MHC class I and a costimulatory molecule are capable of directly inducing T cell cytotoxic activity (Huang et al. 1996). To test whether T cell-mediated tumour cell lysis is inhibited by tumour cell irradiation, purified T cells (1×10^6/ml) were primarily stimulated with irradiated, allogeneous TE.CD80 cells at a final density of 1×10^5/ml. After 6 days of co-culture, T lymphocytes were tested for cytotoxicity against TE.mock (Fig. 16A) or TE.CD80 cells (Fig. 16B). As described (Daniel et al. 1997b), CD80 transgene expression on the target cell slightly enhanced tumour cell lysis as compared to mock transfectants. Prior irradiation did, however, not have a negative impact on the generation CTL-activity ($p > 0.05$ for every E:T ratio tested). Thus, T cell cytotoxicity does not appear to be hampered by irradiation of the tumour cells used for T cell priming.
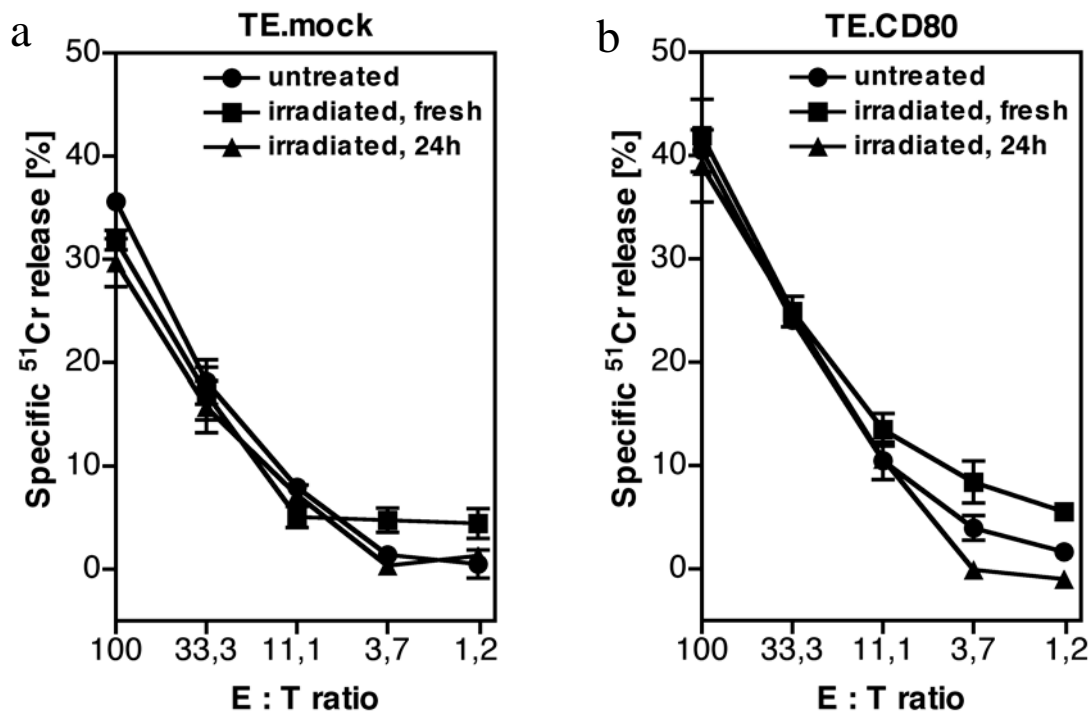

Fig. 16: T cell cytotoxicity. *T lymphocytes prestimulated with irradiated CD80-transfected TE671 cells were restimulated through co-culture with ^{51}Cr-labelled TE.mock (A) or TE.CD80 cells (B) at the indicated effector-target ratios. The tumour cells used for restimulation were either left untreated (circles) or had been irradiated (80 Gy) immediately (squares) or 24 hours (triangles) prior to ^{51}Cr-labelling. After 4 hours of co-culture, ^{51}Cr release from lysed TE.mock or TE.CD80 cells was measured. Representative data from three independent experiments are shown.*

4.9. Induction of T cell proliferation is not affected by tumour cell irradiation

Next, I investigated the effect of tumour cell irradiation on T cell proliferation. T lymphocytes from peripheral blood, enriched by negative depletion to a purity of more than 95%, were stimulated for 18 hours with plastic bound anti-CD3 and anti-CD28 antibodies. Cells were then kept in complete media, containing 100 U/ml recombinant IL-2. Five days after the primary stimulation, T cells were restimulated by CD3 cross-linking in the presence of mock- or CD80-transfected TE671 cells. Tumour cells were either left untreated or had been irradiated (80 Gy) immediately or 24 hours prior to T cell restimulation. After 24 hours of co-culture, T cells were transferred into a fresh 96-well plate and ^{3}H-thymidine was added for an additional 18 hours and T lymphocyte proliferation was measured as ^{3}H-thymidine incorporation. Restimulation of T lymphocytes with anti-CD3 antibodies in the presence of non-irradiated TE.mock cells (7912 cpm ±732) did not increase proliferation as compared to T lymphocytes co-cultured with TE.mock cells and a control IgG antibody (8988 cpm ±2036) (Fig. 17). In contrast, T cell re-activation by CD3 ligation in the presence of TE.CD80 cells significantly enhanced T lymphocyte proliferation (25596 cpm ±2880). Irradiation of tumour cells immediately or 24 hours prior to T cell stimulation did not influence this effect on T lymphocyte proliferation (Fig. 17). Here, T cell proliferation amounted to 23235 cpm (SD ±5117) and 26026 cpm (SD ±5171) upon
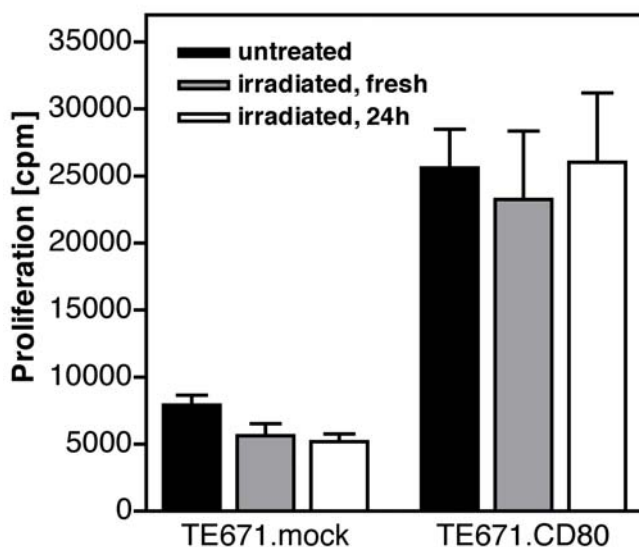

Fig. 17: Induction of T cell proliferation. T cells, pre-activated through CD3 and CD28 ligation, were restimulated by CD3 ligation in the presence of mock- or CD80-transfected TE671 cells. TE671 cells were either left untreated or had been irradiated (80 Gy) immediately or 24 hours prior to co-culture. After 24 hours, T cells were transferred into a fresh 96-well plate and pulsed for 18 hours with 1 μCi ^{3}H-thymidine per well. Proliferation was determined as ^{3}H-thymidine incorporation. Mean values and standard deviations from triplicates are shown.

CD3 ligation in the presence of TE.CD80 regardless of stimulator cell irradiation. Similar results were obtained at other T lymphocyte to tumour cell ratios (data not shown).

4.10. Inhibition of activation-induced cell death is not affected by irradiation

While the costimulatory capacity of CD80-mediated signals has been known for a while, its capability to inhibit AICD has only recently come into focus (Daniel et al. 1997a). To investigate the impact of target cell irradiation on T cell apoptosis, purified activated T cells, pre-activated with anti-CD3 and anti-CD28 antibodies and cultured for 5 days in IL-2 containing medium, were co-cultured with TE.mock transfectants. T cell AICD amounted to 33.3% (SD ±1.15) when T cells were re-activated by anti-CD3 antibody (Fig. 18). As expected, AICD induced by CD3 ligation was effectively inhibited if TE.CD80 transfectants were employed in the co-culture instead of TE.mock cells. In the presence of CD80, AICD decreased to 22.7% (SD ±0.58). Tumour cell irradiation immediately or 24 hours prior to co-culture did not influence the inhibition of AICD by TE.CD80 cells (Fig. 18). Under these conditions, AICD induced by CD3 ligation in the presence of TE.CD80 cells employed immediately or 24 hours after irradiation amounted to 24.0% (SD ±2.65) and 25.7% (SD ±1.15), respectively. Thus, neither induction of T cell proliferation nor inhibition of T lymphocyte AICD are affected by tumour cell irradiation.
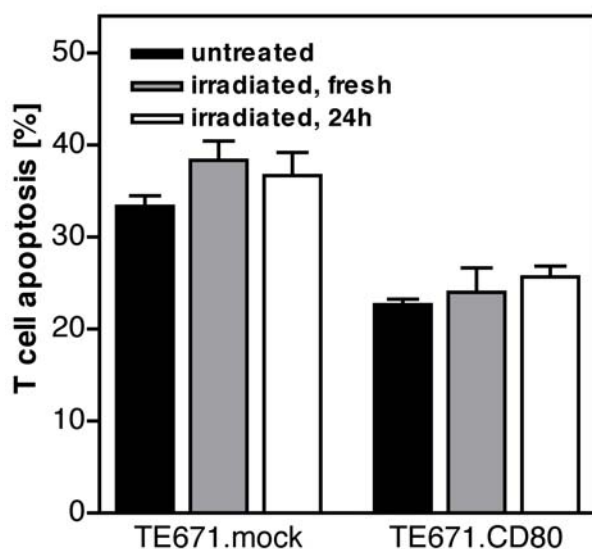

Fig. 18: Inhibition of activation-induced cell death. T cells were activated by CD3 and CD28 ligation and were cultured for 5 days in IL-2 containing medium. These activated T cells were then restimulated by CD3 ligation in the presence of mock- or CD80-transfected TE671 cells. TE671 cells were either left untreated or had been irradiated (80 Gy) immediately or 24 hours prior to co-culture. After 24 hours of co-culture, T cells were transferred into a fresh 96-well plate in order to remove adherent tumour cells. T cells were then cultured another 24 hours and T cell apoptosis was quantified by measuring the nuclear DNA content. Mean values and standard deviations from triplicates are shown.

4.11. Necrotic cell death abolishes the capacity of CD80 expressing tumour cells to costimulate T cell proliferation

Ionising irradiation exerts its cell death inducing effects not only through induction of apoptosis but also via non-apoptotic, necrosis-like cell death. Moreover, instead of inactivation by irradiation, tumour cell vaccines in clinical trials are frequently inactivated through multiple freeze and thaw cycles that result in necrotic cell death. I therefore investigated whether cells inactivated in such a way, i.e. by necrotic cell death, retain their capacity to costimulate T cell effector functions. To this end, TE.CD80 or mock transfectants were inactivated by three freeze and thaw cycles and were co-cultured with pre-activated T cells at a final concentration of 5 x 10^5/ml in the presence or absence of CD3 ligation.

The percentage of apoptotic and necrotic cells was measured by staining cells with annexin-V-FITC and counterstaining with propidium iodide (PI). Apoptosis induces the exposure of phosphatidyl serine groups on the outer leaflet of the cell membrane. These PS groups are recognised by the recombinant, FITC-labelled Annexin-V. Such apoptotic cells cannot be counterstained with PI due to the fact that apoptotic cells avoid permeabilization of the cell membrane. This prevents leakage of cellular constituents that would trigger inflammation and tissue damage. In contrast, necrosis results in the early disintegration of the cell and its membrane. Cells marked with PI as an indicator of membrane damage were counted as being necrotic, while ones that were Annexin-V-FITC positive and PI negative were regarded as being apoptotic. Thus, annexin-V-FITC staining allows for the simultaneous detection of apoptotic and necrotic cell death in mixed populations. The annexin-V-FITC/PI stain reliably measures apoptosis and yields similar results as compared with measurement of DNA fragmentation that occurs in consequence of endonuclease activation during apoptosis (Gillissen et al. 2003). In contrast to LDH release, the uptake of PI by necrotic cells allows the exact assessment of necrotic cell death on the single cell level (Hasenjäger et al. 2004; Friedrich et al. 2001; Wieder et al. 2001). Interestingly, irradiation (80 Gy) of TE.CD80 cells mediated only limited apoptosis or necrosis. In contrast, freeze and thaw treatment induced cell loss by necrosis in more than 95% of the cells examined (Fig. 19A). Prolonged incubation resulted in necrotic death in all cells and coincided with a loss of clonogenic growth. These cells can therefore be considered as irreversibly dead.

Tumour cell inactivation by freeze and thaw cycles completely abolished the tumour cells' capacity to mediate T cell proliferation as determined by ^{3}H-thymidine incorporation (Fig. 19B). As expected and in contrast to tumour cells killed by freeze and thaw cycles, irradiated tumour cells exhibited a low level of background ^{3}H-thymidine incorporation (Fig. 19B, "TE671 alone"). There was no difference, however, between the background proliferation of irradiated TE.mock and TE.CD80 cells.

To elucidate the loss of immune modulatory capacity in necrotic cells, I tested whether cells that had undergone freeze and thaw treatment had lost CD80 expression (Fig. 20). While necrotic cells displayed loss of nuclear integrity and cell fragmentation (Fig. 20A), CD80 expression could still be detected on necrotic cells (Fig. 20B). A
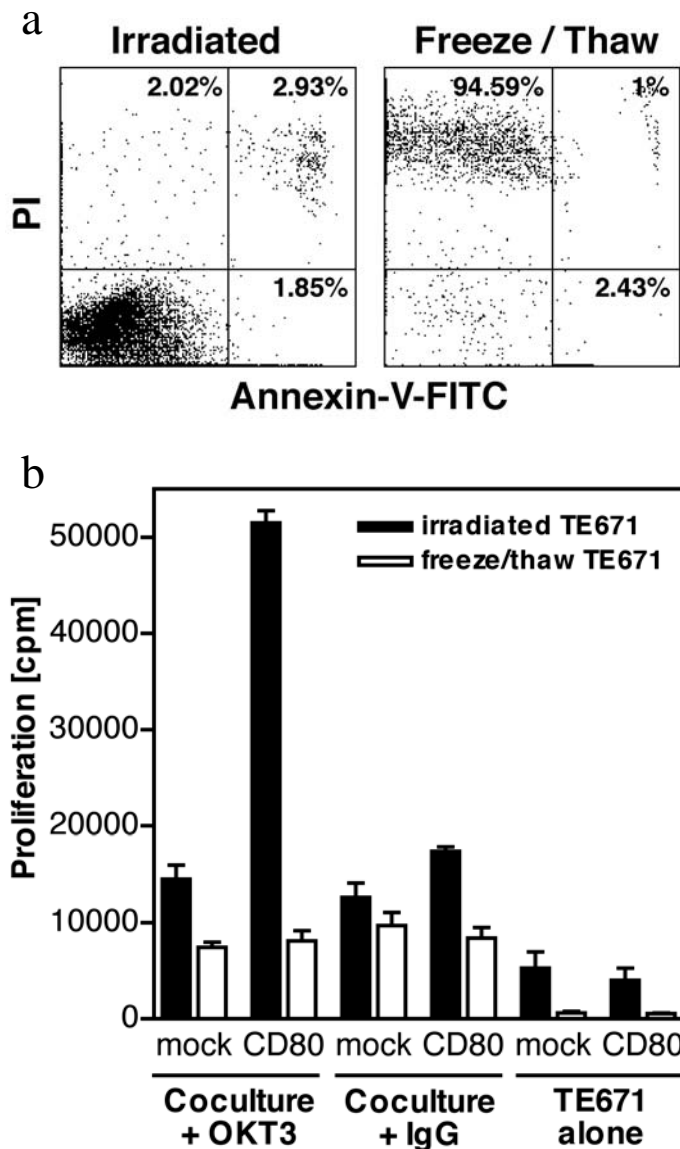

Fig. 19: Costimulatory capacity of TE671 cells inactivated by irradiation or freeze and thaw treatment.
(A). To detect whether irradiation or freeze and thaw treatment mainly induces apoptotic or necrotic cell death, TE671 cells were stained with annexin-V-FITC and counterstained with PI. PI positivity indicates a loss of membrane integrity and is a sign of necrosis, whereas cells positive for Annexin-V, but negative for PI are generally defined as apoptotic. Similar results were obtained on two separate occasions, performed in triplicate.
(B). To measure tumour cell-mediated T lymphocyte proliferation, TE671 cells were either inactivated by ionising irradiation (80 Gy) or exposed to three freeze and thaw cycles. Subsequently, cells were co-cultured with pre-activated T cells in the presence or absence of CD3 ligation. For the last 18 hours of the 42 hour co-culture, cells were pulsed with 1 µCi ^{3}H-thymidine per well. To assess background proliferation, TE671 tumour cells were cultured in absence of T cells. Mean values and standard deviations from triplicates are shown.

quantitative analysis of CD80 expression by flow cytometry showed that the vast majority of necrotic TE.CD80 cells retained CD80 expression as assessed by flow cytometry (Fig. 20C). Thus, necrotic cells still express CD80, although at a slightly broader
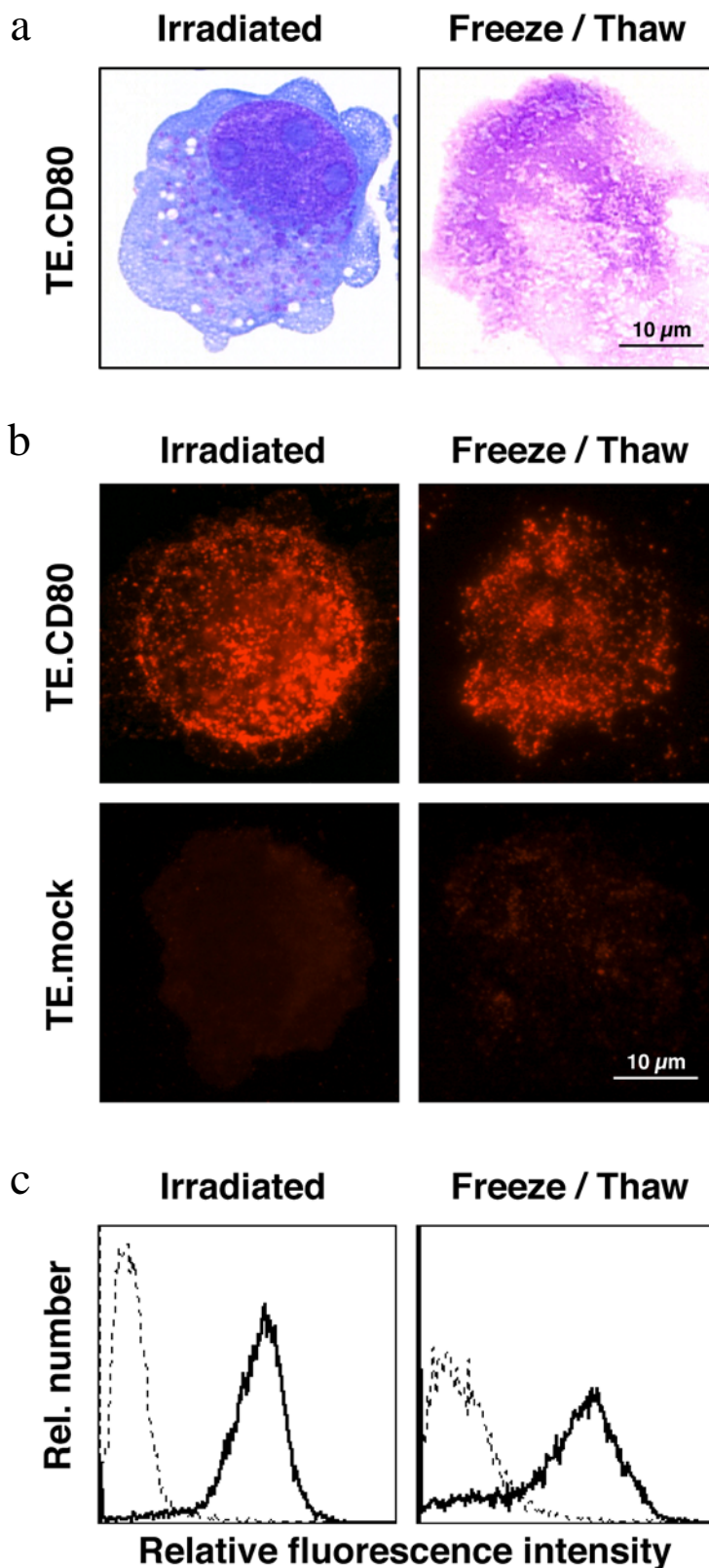

Fig. 20: Morphology and CD80 expression in necrotic TE671 cells.

(A). To assess tumour cells on a single cell level, TE671 cells, previously irradiated or treated with freeze and thaw cycles, were centrifuged onto glass slides, stained with May-Grünwald-Giemsa stain and analysed by light microscopy.

(B). Alternatively, cells were stained with a monoclonal mouse anti-human CD80 antibody followed by Alexa Fluor 594-conjugated chicken anti-mouse IgG. CD80 expression was visualised by UV-light microscopy.

(C). To determine CD80 expression on stably transfected TE671 cells immediately after irradiation or freeze and thaw treatment, tumour cells were stained with a PE-labelled mouse anti-human CD80 antibody (solid line) or a PE-labelled isotype-matched control antibody (dashed line) and analysed by flow cytometry, measuring ten thousand cells from each sample. Cell debris was excluded from the analysis by raising the forward scatter threshold. For the analysis, cells remained ungated. A representative of three separate experiments is shown.

distribution and occurrence of a few negative cells, possibly due to protein turnover and impaired resynthesis. Lack of costimulatory capacity was, therefore, not due to a reduction of transgene expression during the short term culture.

4.12. Irradiation of tumour cells induces necrosis depending on the cell line assessed

While apoptotic cell death has been reported to be the predominant mode of cell death after irradiation (Shinomiya 2001; Harms-Ringdahl, Nicotera & Radford 1996; Zhou, Yuan & Serggio 2003), recent data demonstrated that the cytotoxic activities of ionising irradiation also lead to necrosis (Kotera, Shimizu & Mule 2001). To assess the amount of necrosis induced in my system, cells from the rhabdomyosarcoma cell line TE671 were irradiated with a single dose of 20 or 80 Gy. The percentage of ap-
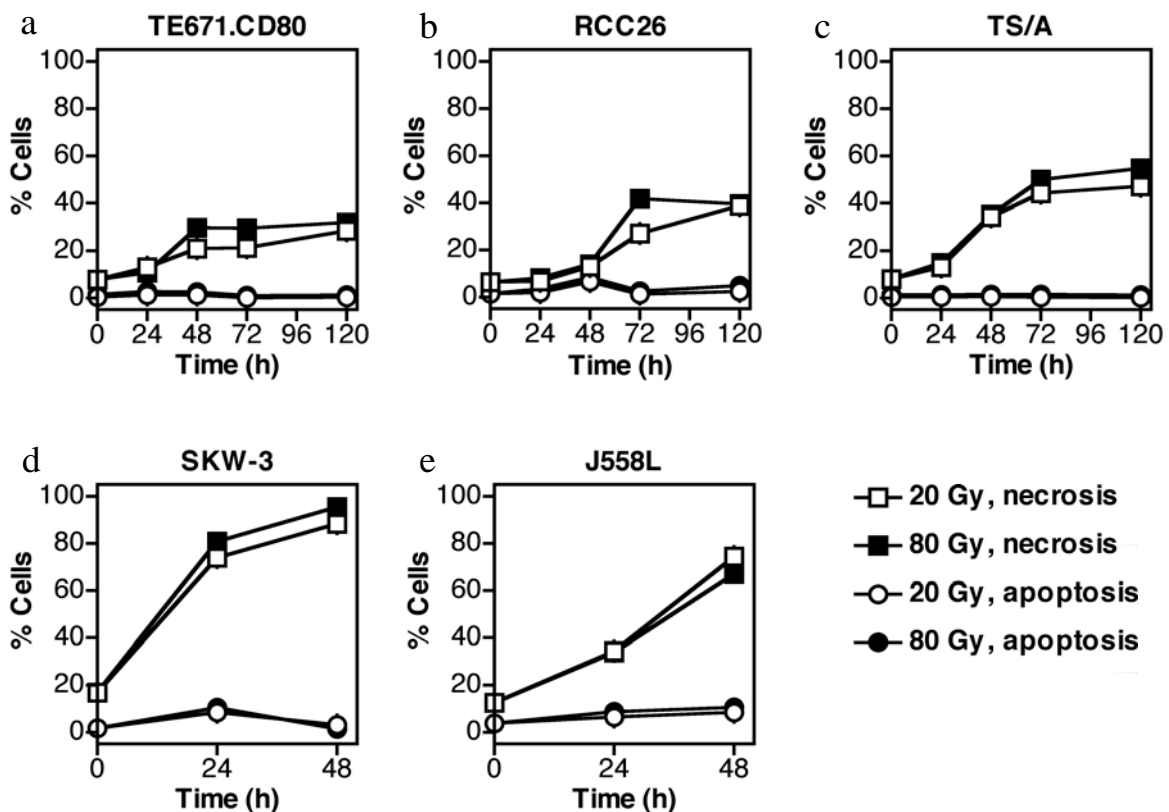

Fig. 21: Irradiation-mediated apoptosis and necrosis of tumour cell lines. (A-E). Tumour cell lines, as indicated, were irradiated with 20 Gy (open squares and circles) or 80 Gy (solid squares and circles). Cells were double-stained with annexin-V-FITC/PI and analysed by flow cytometry at the indicated time points. Annexin-V single positive cells were counted as apoptotic, while PI single or PI/annexin-V double positive cells were counted as necrotic. The percentages of apoptotic cells are depicted as open or solid circles, whereas the percentages of necrotic cells are shown as open or solid squares. Mean values and standard deviations from triplicates are shown.

optotic and necrotic cells was measured at distinct time points by staining cells with annexin-V-FITC and counterstaining with PI to assess loss of membrane integrity as indicator of necrotic cell death. Notably, the amount of necrotic cells only moderately increased from 7.79% (SD ±1.2) before irradiation, to 10.82% (SD ±1.24), 29.73% (SD ±1.31), 29.5% (SD ±0.32) and 32.05% (SD ±0.676) 24, 48, 72 and 120 hours after irradiation with 80 Gy, respectively (Fig. 21A). In contrast, more than 95% tumour cell necrosis was induced through 3 freeze and thaw cycles (Fig. 19A).

To test whether irradiation at dosages commonly applied to inactivate tumour cell vaccines generally mediates only moderate amounts of necrosis, different tumour cell lines were tested. To this end, RCC26, a human renal cell carcinoma, SKW3, a human B lymphoid cell line derived from a patient with chronic lymphocytic leukaemia, TS/A, a murine mammary adenocarcinoma cell line and J558L, a murine plasmacytoma cell line, were selected. The latter two cell lines have been employed successfully as cellular vaccines in murine tumour models (Cayeux et al. 1995, 1996; Hock et al. 1993a). There, Cayeux et al. demonstrated, however, that irradiated TS/A and J558L tumour cells, expressing cytokines and/or CD80, mediated a much weaker vaccine effect as compared to living tumour cells. When assessing the amount of necrosis induced by 20 and 80 Gy of irradiation, I was intrigued to find that levels of necrosis varied largely between the cell lines tested (Fig. 21 A-E). In line with this, both J558L and TS/A cells responded with a high degree of necrotic cell death that was even more pronounced in the lymphoid cell line SKW3. In contrast, the renal cell carcinoma line RCC26 was, like the rhabdomyosarcoma TE671, highly radioresistant. All cell lines showed only background levels of apoptotic cell death at these early time points. This significant rate of necrotic cell death might account for the loss of costimulatory activity of gene-modified tumour cells described in some preclinical models upon ionising irradiation.