5. Discussion

5.1. Costimulatory signals regulate T cell proliferation and apoptosis

To date, research on costimulatory molecules has mainly focused on two protein families. While CD28, ICOS and CTLA-4 belong to the immunoglobulin superfamily, 4-1BB and OX40 are part of the tumour necrosis factor receptor family.
In my study I compared the costimulatory capacities of CD28 and 4-1BB in respect to their ability to inhibit AICD in primary human T lymphocytes. Here, I focused on intracellular signal pathways that mediate apoptosis protection. Therefore I used an *in vitro* model that represents the main features of T cell reactivity: Freshly purified human T lymphocytes, polyclonally stimulated with immobilised antibodies to CD3/CD28 and cultured for a prolonged time in IL-2 containing medium represent AICD sensitive T cells at the down phase of an immune response (Hedfors & Brinchmann 2003). These cells were restimulated by antibodies to CD3, co-cultured in the absence of exogenous IL-2 with tumour cell lines stably transduced to express CD80, 4-1BBL or a mock control and then further analysed.
Here, I demonstrate that costimulatory signals through CD28 or 4-1BB induce proliferation and inhibit AICD upon T cell restimulation. I showed for the first time that c-FLIP$_S$, a major inhibitor of the extrinsic pathway of apoptosis, was up-regulated following 4-1BB-mediated costimulatory signals. Equally, Bcl-x$_L$, a key inhibitor of the mitochondrial pathway of apoptosis, was up-regulated under either condition. When analysing the signalling pathways involved, costimulation through CD28 or 4-1BB augmented TCR/CD3-mediated AKT phosphorylation. In addition, blockade of PI3 kinase or of AKT completely abolished CD28- and 4-1BB-mediated T cell proliferation, AICD inhibition, AKT phosphorylation and up-regulation of Bcl-x$_L$ and c-FLIP$_S$. This suggests that CD28- and 4-1BB-mediated signals share a common signalling pathway which involves PI3 kinase activation and AKT phosphorylation as necessary events.

5.1.1. Costimulatory signalling through 4-1BB

While signalling pathways induced through CD28 ligation in conjunction with TCR/CD3-mediated signals are well defined, pathways involved during 4-1BB-mediated AICD inhibition are less well understood. To date, reports showed expression of

4-1BB, a member of the tumour necrosis factor receptor family, on activated CD4 and CD8 T cells (Pollok et al. 1993; Alderson et al. 1994), differentiated dendritic cells (DeBenedette et al. 1997), activated B cells and activated macrophages (Alderson et al. 1994). Ligation of 4-1BB is known to costimulate CD3-mediated T cell activation, cytotoxicity (Melero et al. 1997; Shuford et al. 1997; Tan et al. 2000) and cytokine secretion (Wen, Bukczynski & Watts 2002). In addition, 4-1BB has been reported to inhibit AICD of activated T cells (Hurtado, Kim & Kwon 1997).

A recent report showed that cross-linking of 4-1BB molecules recruit 4-1BB, TRAF-2 and the TCR signal molecules Lck, pTyr, PKC-θ and SLP-76 into lipid rafts and increases intracellular Ca^{2+} levels (Nam et al. 2005). Also it has been shown that ligation of 4-1BB results in recruitment of TRAF-1 and -2 to the cytoplasmatic domain of 4-1BB (Arch & Thompson 1998). TRAF-2 in turn mediates 4-1BB-induced NF-κB activation (Arch & Thompson 1998) via NF-κB inducing kinase (NIK) (Natoli et al. 1997; Akiba et al. 1998) and Bcl-x_L is a well established transcriptional target of NF-κB (Chen, Edelstein & Gelinas 2000; Khoshnan et al. 2000). AKT-dependent induction of Bcl-x_L via NF-κB has been demonstrated in a transgenic mouse model (Jones et al. 2000). This may well explain the mode of Bcl-x_L induction through 4-1BB costimulation in the present setting, as 4-1BB-induced Bcl-x_L up-regulation is abolished in the presence of PI3 kinase and AKT inhibitors.

In a different publication, however, T cell survival mediated by 4-1BB, mainly depended on NF-κB activity. There, inhibition of NF-κB with the antioxidant 1-pyrrolidinecarbodithioic acid (PDTC) decreased the percentage of surviving T cells and diminished Bcl-x_L and Bfl-1, while blockade of PI3 kinase by use of the inhibitor LY 294002 had a less pronounced effect but nevertheless interfered with T cell survival and Bfl-1 and Bcl-x_L expression (Lee et al. 2002). Besides, a close look at their western blot data reveals a weaker Bcl-x_L band when the PI3 kinase inhibitor is present. Furthermore, their experimental setup is not directly comparable to mine, as they employ murine CD8 T cells and examine a primary stimulation rather than a restimulation. These differential effects of PDTC and PI3 kinase inhibition on Bcl-x_L and survival might also be due to unspecific effects of the antioxidant PDTC or might simply point out PI3 kinase independent activation NF-κB signalling. For example, 4-1BB-induced IL-2 expression could induce STAT5 (signal transducers and activators of transcription) via the IL-2 receptor in an auto- or paracrine fashion (Lin & Leonard 2000; Benczik &

Gaffen 2004) and in turn mediate Bcl-x_L up-regulation independent of PI3 kinase (Grad, Zeng & Boise 2000; Calo et al. 2003). Furthermore, the signal complex between TRAF and 4-1BB has also been shown to activate JNK/SAPK via p38 MAPK (Cannons et al. 1999) and to facilitate activation of NF-κB (Arch & Thompson 1998). I observed 4-1BB costimulation-induced proliferation which is dependent on the PI3 kinase/AKT pathway. In line with my data, Lee et al. could inhibit 4-1BB costimulation-induced proliferation with the PI3 kinase inhibitor LY 294002 (Lee et al. 2002, 2003). Here, they show that 4-1BB elicits up-regulation of the cell cycle regulators cyclin D2, D3 and E through ERK1/2 and PI3 kinase pathways. This in turn inhibits expression of the cdk inhibitor $p27^{kip1}$ and leads to cell cycle progression, which further explains my findings (Lee et al. 2003).

Apart from Bcl-x_L up-regulation, I observed also an increase of c-$FLIP_S$ expression though 4-1BB-mediated costimulation. Same like Bcl-x_L, c-$FLIP_S$ up-regulation was PI3 kinase- and AKT-dependent. Recent data demonstrated that phosphorylated AKT regulates the expression of the anti-apoptotic protein c-FLIP by use of distinct pathways. To date, it has been published that phosphorylated AKT inhibits FOXO3a, a transcription factor that mediates, for example, down-regulation of c-FLIP (Skurk et al. 2004). Aside from inducing Bcl-x_L expression, NF-κB has been shown to elicit up-regulation of c-FLIP in T cells, conferring to resistance to FasL and TNF-mediated apoptosis (Micheau et al. 2001; Mora et al. 2003). Several publications report PI3 kinase or AKT-dependent c-FLIP up-regulation in tumour cells (Panka et al. 2001; Nam et al. 2003) or endothelial cells (Suhara et al. 2001). Also, induction of PI3 kinase and phosphorylation of AKT have been reported for the TNFR family member OX40 (Croft 2003). Taken together with the above discussed observation that 4-1BB can activate the PI3 kinase/AKT pathway, this supports my findings regarding PI3 kinase and AKT dependency in 4-1BB-mediated c-$FLIP_S$ up-regulation.

It is known that c-$FLIP_S$ is recruited to the DISC and rescues cells from AICD (Scaffidi et al. 1999; Thome & Tschopp 2001), which is believed to be mainly CD95-mediated (Alderson et al. 1995). In line with this, I observed AICD inhibition when costimulation was present, i.e. when c-$FLIP_S$ levels were high. Also, AICD inhibition was PI3 kinase- and AKT-dependent.

5.1.2. Costimulatory signalling through CD28

Signalling pathways induced by CD3 and CD28 ligation have been reported in great detail, i.e. ligation of CD28 induces tyrosine phosphorylation within its cytoplasmatic tail and subsequent PI3 kinase activation (Pages et al. 1994; Prasad et al. 1994). PI3 kinase on its part generates phosphatidylinositol-3,4,5-triphosphate (PIP_3), a lipid second messenger that acts as a binding site for numerous enzymes that contain pleckstrin homology domains (Okkenhaug & Vanhaesebroeck 2003), for example AKT (Parry et al. 1997; Vanhaesebroeck & Alessi 2000; Kane et al. 2001), PKC, PDK1 or Tec (Chan, Rittenhouse & Tsichlis et al. 1999). As discussed above (see section 5.1.1., page 63), phosphorylated AKT induces NF-κB and inhibits FOXO3a, leading to up-regulation of Bcl-x_L and c-$FLIP_S$.

In line with my data presented here, it has been reported that CD28-mediated signals, in conjunction with TCR/CD3 ligation, induce phosphorylation of AKT (Kane et al. 2001), promote T cell survival and induce Bcl-x_L (Boise et al. 1995) and c-$FLIP_S$ expression (Kirchhoff et al. 2000). Furthermore, AKT activation has been described after stimulation through TCR/CD3 as well as after ligation of CD28 itself (Parry et al. 1997; Lafont et al. 2000). It is therefore of note that I observed a transient and weak but reproducible up-regulation of c-$FLIP_S$ at 6 hours after restimulation by CD3 ligation itself. This transient AKT phosphorylation and c-$FLIP_S$ up-regulation after CD3 ligation alone in the absence of CD28 or 4-1BB ligation did not suffice to facilitate T cell proliferation or AICD inhibition. Therefore, a more pronounced AKT phosphorylation might be required to provide a sustained anti-apoptotic c-$FLIP_S$ signal which in turn would suffice to inhibit AICD.

Elsewhere, it was demonstrated that employment of the PI3 kinase inhibitors wortmannin at a dosage of up to 500 nM or LY 294002 at a dosage of maximally 5 µM facilitated a partial down-regulation of Bcl-x_L but failed to reverse CD28-mediated rescue from AICD (Collette et al. 1997). However, for primary human T cells, I observed in my system a substantial inhibition of the CD28-induced rescue from AICD at LY 294002 concentrations of 10 and 20 µM. At 10 µM LY 294002 Bcl-x_L and c-$FLIP_S$ were completely down-regulated as assessed by Western blot. Therefore, one might speculate that a higher dosage of LY 294002 is needed to block both the expression of Bcl-x_L and $FLIP_S$.

5.1.3. Costimulatory signalling and AICD inhibition

In my study, costimulation-induced up-regulation of the anti-apoptotic proteins Bcl-x_L and c-$FLIP_S$, which act at the intrinsic or extrinsic death pathway, respectively. It can be speculated if one of these molecules has a more pronounced function or if they cooperate in AICD inhibition. Scaffidi et al. described two different CD95 pathways, designated type I and type II (Scaffidi et al. 1998). In type I cells CD95 triggering leads to strong caspase 8 activation at the DISC which bypasses mitochondria, directly leading to activation of other caspases such as caspase 3 and subsequently to apoptosis. In contrast, DISC formation is reduced in type II cells and, therefore, only small amounts of procaspase 8 are recruited and activated at the DISC. Here, induction of apoptosis depends on the mitochondria as amplifiers for downstream death substrates. Therefore, blocking activation of mitochondria by Bcl-2 or Bcl-x_L inhibits apoptosis only in type II cells. FLIP, in contrast can inhibit apoptosis both in type I and type II cells (Scaffidi et al. 1998, Schmitz et al. 2003). Freshly activated T cells are reported to behave like type II cells, whereas prolonged culture with IL-2 transforms them into type I cells (Peter et al. 1997; Schmitz et al. 2003). This would imply that c-$FLIP_S$ up-regulation mediates the anti-apoptotic effect observed in my system.

To further confirm this hypothesis, c-$FLIP_S$ and Bcl-x_L would have to be inhibited or overexpressed selectively, which is difficult to establish in primary human T cells. In a transgenic mouse model it was shown that CD8 T cell apoptosis in the down-phase of an immune response against lymphocytic choriomeningitis virus (LCMV) is not prevented by Bcl-x_L or Bcl-2 expression (Petschner et al. 1998). Here, the authors conclude that two distinct apoptosis pathways exist in CD8 T cells of which one can not be blocked by Bcl-x_L or Bcl-2. Expression of c-$FLIP_S$ in their system could further elucidate its role in AICD inhibition.

My study revealed a central role for AKT in mediating T cell survival. Also, AKT has been shown to antagonise pro-apoptotic functions of Bad and caspase 9 through phosphorylation (Datta et al. 1997; Cardone et al. 1998). Furthermore, it is reported that AKT can induce NF-κB (Kane et al. 1999), which regulates IL-2 transcription (Yamamoto & Gaynor 2001). IL-2 seems to be important for priming T cells to AICD (Peter et al. 1997; Refaeli et al. 1998; Schmitz et al. 2003). In murine T cells AICD was enhanced by IL-2 and an IL-2-dependent c-$FLIP_L$ down-regulation was observed

(Refaeli et al. 1998). However, in line with my observations, it is reported that human T lymphocytes do not down-regulate c-FLIP$_L$ in response to IL-2 (Scaffidi et al. 1999), ruling this out as a mechanism for IL-2-mediated AICD sensitisation.

Further investigation into the role of PI3 kinase or AKT activation in relation to NF-κB activation and other signalling events initiated by 4-1BB and CD28 ligation should yield interesting insights into the regulation of T cell survival and costimulation. Notably, ligation of CD28 during T cell receptor stimulation has been reported to augment JNK/SAPK activation induced by suboptimal amounts of anti-CD3 antibody (Rivas, O'Herrin & Gajewski 2001). Furthermore, IL-4, IL-5 and IFN-γ, but not IL-2 secretion following CD28 costimulation in human T cells depended on p38 MAPK activation (Koprak, Staruch & Dumont 1999; Schafer et al. 1999a, 1999b). Thus, key proteins from both major costimulatory molecule families appear to be involved in multiple additional signalling pathways.

It is of note, however, that my data does not formally demonstrate a direct interaction between 4-1BB and PI3 kinase. I can not rule out that PI3 kinase is induced by cytokines such as IL-2, which in turn are secreted in response to 4-1BB-mediated NF-κB activation. The absence of the PI3 kinase binding motif Tyr(P)-Met-Xaa-Met (Prasad et al. 1994) in 4-1BB furthermore argues against a direct interaction between 4-1BB and PI3 kinase. However, there is data indicating that signals from TNFR family members, i.e. 4-1BB or OX40, facilitate PI3 kinase and AKT activation, at least to some extend independent of cytokines. In this vein, a recent publication (Song et al. 2004) demonstrated that OX40-mediated signals induce sustained AKT phosphorylation. Interestingly, the authors observed that exogenous IL-2 did not sustain AKT activation in a similar manner in OX40 deficient cells, thus demonstrating the need for OX40-mediated signals (Song et al. 2004). OX40 and 4-1BB play similar, yet not identical roles in costimulation (Croft 2003). For the TNFR family member CD40, proteins like Cbl appear to mediate interaction with PI3 kinase (Arron et al. 2001). The involvement of 4-1BB-mediated signals in PI3 kinase and AKT activation is, furthermore intriguing as the OX40-mediated, sustained AKT phosphorylation is presumed to facilitate T cell longevity (Song et al. 2004). Regarding my data, one might speculate that the observed 4-1BB-mediated AKT phosphorylation might play a similar role.

As 4-1BB-induced signals appear to play a key role during late primary and during secondary T cell responses they might be more important for a sustained T cell response than CD28-mediated stimuli (Bertram et al. 2004; Dawicki & Watts 2004). In this vein, it is of note that I observed a moderate increase of proliferation and a fair decrease of apoptosis in pre-activated T lymphocytes upon co-culture with the 4-1BBL expressing transfectants, in the absence of a CD3-mediated signal, as compared to T cells that were cultured with mock transfectants alone (Fig. 10). Such a change of proliferation, notably to a lesser extend, was furthermore detected if pre-activated T cells were cultured with CD80 expressing transfectants alone. One may speculate that on day five after the primary activation a certain percentage of T cells is still activated enough to be driven into proliferation or saved from apoptosis by a costimulatory signal alone. In line with this, proliferation of pre-activated T cells through CD28 ligation in the absence of a CD3-mediated signal has been described before (Siefken et al. 1998). 4-1BB-derived signals, however, have been shown to be more important during the late primary and secondary T cell stimulation (Bertram et al. 2004; Dawicki & Watts 2004). Thus, the percentage of pre-activated T cells capable of proliferating or surviving to a costimulatory signal alone might be higher for 4-1BB as compared to CD28 stimulated T cells.

In conclusion, my data demonstrate that 4-1BB- and CD28-mediated costimulatory signals share downstream components, i.e. members of the PI3 kinase and AKT signalling pathway. Furthermore, I show that AKT activation is a required condition for 4-1BB-mediated inhibition of AICD and up-regulation of Bcl-x_L and c-$FLIP_S$. The notion of shared downstream signalling pathways is especially interesting as signals mediated through CD28 or 4-1BB appear be of importance during different stages of T cell responses, i.e. during primary T cell activation as opposed to secondary T cell responses and sustained T cell activity, respectively.

5.2. Costimulatory signalling in tumour immunology

Cellular cancer vaccines have been employed to treat minimal residual disease in various preclinical models and are currently evaluated in clinical trials (Yannelli et al. 1993; Bowman et al. 1998; Simons et al. 1999; Vermorken et al. 1999; Kusumoto et al. 2001; Takahashi et al. 2001; Graf et al. 2002; Rousseaut et al. 2003; Jocham et al. 2004). It is, however, unknown whether the maximal tumour-specific T cell response is achieved by living tumour cells or by cell vaccines inactivated through irradiation or freeze and thaw treatment. Here, I show that living tumour cells and tumour cells inactivated by irradiation induce similar T cell effector responses, i.e. T cell proliferation, inhibition of AICD and tumour cell lysis. In contrast, the induction of necrosis through freeze and thaw treatment completely abolished the tumour cells' capacity to mediate *in vitro* T cell activation.

Data from murine tumour models demonstrated that tumour rejection upon cancer vaccination is largely T cell-dependent (Hock et al. 1993b; Cayeux et al. 1995; Willimsky & Blankenstein 2000). Apart from indirect T cell activation through presentation of tumour peptides on APC, tumour cells, frequently expressing MHC class I, are capable of directly triggering tumour specific CTLs, for example, when they express CD80. However, signalling through the TCR in the absence of a costimulatory signal renders naïve T cells anergic and mediates activation-induced cell death in pre-activated T cells (Daniel et al. 1997a, 1999). In contrast, genetic engineering of tumour cells with costimulatory ligands, for example CD80 and/or cytokines, for example IL-7, helps to circumvent T cell anergy and facilitates a potent T cell response in transplanted and non-transplanted tumour models (Huang et al. 1996; Cayeux et al. 1997; Schendel et al. 2000). Furthermore, AICD which is mediated through ligation of death receptors, for example CD95, on activated lymphocytes can be inhibited by CD80 expressing tumour cells (Dhein et al. 1992; Daniel & Krammer 1994; Daniel et al. 1997a). In line with this, my results demonstrate that expression of the costimulatory ligand CD80 in TE671 tumour cells improved T cell effector functions as compared to mock transfected tumour cells.

While the increase of immunogenicity through transgene expression has been observed by various groups, it is largely unclear whether immune modulated tumour cells have to be alive to induce maximal T cell activity. This question appears to be especially important as tumour cell vaccines for humans are often employed in a situa-

tion where the primary tumour and sometimes even tumour metastases have been surgically removed. In such a situation tumour vaccines are aimed at the putatively remaining minimal residual disease (MRD). The use of viable cells as vaccines in such a clinical setting stands the risk of survival of the injected cancer cells and therefore bears the danger of tumour implantation. To this end, the accidental transplantation of a malignant sarcoma from a patient to a surgeon through an injury inflicted to the surgeon during the patients emergency surgery, has been reported (Gärtner et al. 1996). Consequently, tumour cell vaccines for humans are typically inactivated. Cell killing for clinical trials can be achieved by lethal ionising irradiation or inducing necrosis through multiple freeze and thaw cycles. Nevertheless, the evidence for the efficacy of such lysates is questionable. Therefore, in most clinical trials tumour cell vaccines are inactivated by irradiation with single dosages ranging from 50 to 200 Gy. Irradiation is thought to induce mainly apoptosis (Harms-Ringdahl, Nicotera & Radford 1996; Shinomiya 2001; Zhou, Yuan & Serggio 2003), characterised by distinct morphological changes including membrane blebbing, cellular shrinkage and chromatin condensation (Daniel et al. 2001). Nevertheless ionising radiation may also result in non-apoptotic, necrotic cell death.

A number of groups have compared the immunogenicity of apoptotic and necrotic tumour cell vaccines often concluding that apoptotic cells induce a stronger *in vivo* and *in vitro* immune response as compared to necrotic cells (Schnurr et al. 2002; Scheffer et al. 2003). This supports the use of irradiated tumour cell vaccines in clinical studies. There is, however, data demonstrating the reverse, i.e. necrotic tumour cells are more immunogenic than apoptotic tumour cells (Melcher et al. 1998). This view is supported by observations where necrotic cells but not apoptotic cells are capable of releasing heat shock proteins, which in turn activate professional antigen presenting cells (Basu et al. 2000). The notion that cell death through necrosis, as opposed to cell death through apoptosis, facilitates the activation of the immune system is part of the so called "danger model" (Gallucci & Matzinger 2001). While some controversy regarding the immunogenicity of necrotic as compared to apoptotic cells remains, there are little data comparing apoptotic to living tumour cell vaccines. To this end, the immune priming capacity of a non-inactivated, gene-modified tumour cell vaccine expressing CD80 and IL-7 was compared to the same cellular vaccine previously irradiated with 50 or 100 Gy. Vaccination with living, gene-modified tumour cells completely protect-

ed mice from tumour growth upon rechallenge with non-transfected cells. In contrast, irradiated tumour cell vaccines provided a less complete protection. Furthermore, tumour rejection was dosage-dependent. While 70% (7 of 10 mice) developed a tumour upon rechallenge if the vaccine was irradiated with 100 Gy, only 38% (3 of 8 mice) did so after an irradiation with 50 Gy (Cayeux et al. 1995). Similar results were observed when viable or irradiated (100 Gy) tumour cell vaccines, modified to express CD80/IL-4 were employed (Cayeux et al. 1996). These results led the authors to speculate that irradiation of vaccine cells almost completely abolishes their immunization effect. Therefore, when setting out to investigate the impact of irradiation on T cell activation and costimulation *in vitro*, I was surprised to find that key T lymphocyte effector functions, i.e. proliferation, avoidance of AICD and tumour cell lysis, remained unchanged after irradiation of tumour cells. This implies that costimulatory signals by gene-modified tumour cells and their propensity to activate T cells is not disturbed by irradiation. My notion of preserved immunogenicity of irradiated tumour cells is supported by a recent publication demonstrating that human dendritic cells induced maximal T cell proliferation and CTL cytolytic activity for tumour targets when pulsed with apoptotic instead of necrotic or living cells. This demonstrates that the ability for *in vitro* cross-priming of T lymphocytes is preserved after irradiation (Shaif-Muthana et al. 2000). This and the here presented observation are further corroborated by a report where living and irradiated tumour cell vaccines gene-engineered to express the granulocyte-macrophage colony stimulating factor had similar vaccine potency irrespective of irradiation (Dranoff et al. 1993). Finally, ionising irradiation triggers a cellular stress response that might further enhance immunogenicity of the damaged cell whereas freeze and thaw inactivation should not induce gene expression in the necrotic cell. Moreover, even lethally irradiated cells may undergo a few cell cycles that can result in a limited expansion of the antigen and costimulation available for immune stimulation. In contrast, necrosis induced by freeze and thaw physically kills the stimulator cell and, due to the irreversible and rapid death induced by this procedure, such necrotic cells might persist for shorter time periods as compared with cells that were inactivated, for example, by lethal irradiation. Such variations in the kinetics of vaccine persistence may add *in vivo* to the observed failure of necrotic CD80 transfectants to costimulate T cell responses.

A number of reasons could be responsible for this discrepancy. While loss of transgene expression as the reason for the reduced immunization effect has been ruled out by Cayeux et al. (Cayeux et al. 1995) and my present data, one may speculate that the susceptibility for irradiation-induced necrosis could account for the differences observed. To this end, a direct comparison of the TE.CD80 cells, used in my model, to TS/A and J558L, the cell lines employed in experimental tumour vaccination by Cayeux et al. (Cayeux et al. 1995, 1996), demonstrated that the latter two cell lines were more susceptible to irradiation-induced necrosis. Similarly, a higher rate of necrotic death upon irradiation was observed in RCC26 that is under current investigation as a clinical vaccine in renal cell cancer. It is worth noting that necrotic cells, as compared to living cells, do not proliferate *in vivo* after vaccination. One might therefore speculate that living tumour cell vaccines facilitate optimal amounts of antigen to successfully prime the immune system, while necrotic tumour cells fail to do so.

Finally, I demonstrated here that TE.CD80 cells, rendered necrotic by freeze and thaw cycles, completely lost their capacity to activate purified T lymphocytes *in vitro*. My present data clearly therefore indicate that necrotic cell death abrogates the costimulatory potency of CD80 expressing cells. Notably, necrosis did not significantly affect the level of CD80 expression. Recent data illustrated, however, that cell-cell interactions are fundamental to the initiation of strong T cell responses (Davis et al. 2003). Rendering tumour-target cells necrotic therefore possibly impedes the segregation of costimulatory molecules, for example CD80, into the formation of cellular synapses between tumour cell and the T lymphocyte.

In conclusion, my observation expands existing *in vitro* and *in vivo* data and shows that irradiation of tumour cell vaccines does not interfere with their propensity to induce T cell activation for proliferation, cytotoxicity and protection from T cell apoptosis. In contrast, necrotic cells failed to induce these functions. Therefore, employment of irradiated tumour cell vaccines avoiding the risk of inoculating additional tumour burden has the potential to initiate the desired T cell stimulatory effects. However, the amount of necrosis induced by irradiation might negatively influence the vaccination effect and must be taken into account in vaccine development.