1. Introduction

The immune system of a multicellular organism has several functions. It acts primarily as a defence against foreign pathogens and possibly eliminates internal threats such as cancer (Blattman & Greenberg 2004). T cells play a key role in immunity as they distinguish self from non self and regulate humoral and cellular immune responses. To be activated, T cells require an antigen specific signal through the T cell receptor (TCR) and a second, costimulatory signal (Bretscher & Cohn 1970). Signalling events that follow the ligation of CD28, a member of the immunoglobulin superfamily, have been described to a large extent. In contrast, less is known about costimulatory signals through mediators of the tumour necrosis factor receptor (TNFR) family, for example 4-1BB/CD137.

The first part of the thesis investigates molecular events mediated by 4-1BB ligation. These studies were carried out employing tumour cells, genetically modified to express the costimulatory ligands of 4-1BB or CD28, i.e. 4-1BB ligand (4-1BBL) or CD80, respectively.

The second part of the thesis deals with tumour cell vaccines in general. For usage in cancer patients with minimal residual disease, tumour cell vaccines have to be inactivated, for example, by irradiation or repeated freeze and thaw cycles. There is evidence from mouse models that inactivation might jeopardise the vaccine's capacity to modulate the immune system. Here, I compare the capacity of tumour cells, inactivated through irradiation or freeze and thaw treatment, for direct T cell activation.

1.1. Costimulatory signals regulate T cell proliferation and apoptosis

T cells require a T cell receptor-mediated and a costimulatory signal for the initiation of T cell effector functions (Plas, Rathmell & Thompson 2002). The first signal is provided by engagement of the TCR with its specific peptide antigen, bound to MHC molecules on the surface of antigen presenting cells (APC). The second signal is mediated by interaction of costimulatory receptors on the T cell surface with their specific ligands on APC. The coordinated triggering of these two independent receptor systems ensures the correct interpretation of the TCR-mediated signal and induces production of the autocrine growth factor interleukin-2 (IL-2), leading to proliferation of effector T cells.

In contrast, TCR-mediated stimulation of naïve T cells in the absence of a co-stimulatory signal through CD28 induces T cell anergy and is likely to be involved in T cell tolerance, for example, against tumour cells (Gimmi et al. 1993; Pardoll 1998; Frauwirth & Thompson 2002). If a naïve T cell receives only a signal through the TCR it will become anergic (Fig. 1). Anergic T cells do not produce IL-2 upon re-exposure to antigen (even in the presence of costimulation), but the anergic state can be reversed by the addition of exogenous IL-2 (Appleman & Boussiotis 2003). Interestingly, not all costimulatory molecules provide a positive signal that leads to proliferation and expansion; some, for example CTLA-4, provide a negative signal that results in termination of immune responses (Van Parijs & Abbas 1998).

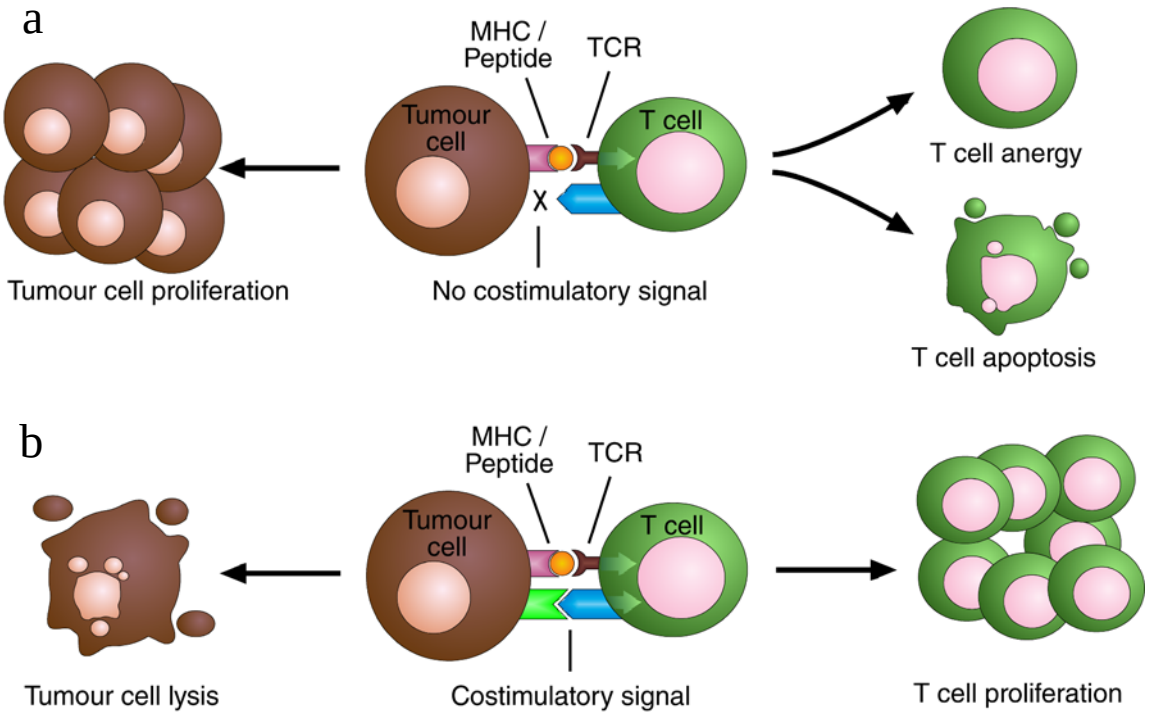

Fig. 1: T cell activation. *(A) Antigen presentation in the absence of costimulatory signals induces T cell anergy in naïve T cells and activation-induced cell death of previously activated T cells. (B) Antigen presentation in conjunction with costimulatory signals induces T cell proliferation and T cell effector functions, which, for example, can promote tumour cell lysis.*

1.1.1. Costimulatory members of the CD28 receptor family

The CD28 receptor family is a subfamily of the immunoglobulin (Ig) superfamily. Members of this superfamily share a common evolutionary precursor, the immunoglobulin homology unit, and are found in many molecules of the immune system and in molecules of no known immunological functions. The CD28 family members

presented below share great sequence homology and are disulfide-linked homodimers in which each chain has a single extracellular variable-like domain (Hutloff et al. 1999, Ostrov et al. 2000, Aruffo & Seed 1987).

1.1.1.1. CD28

So far, CD28 is the best studied T cell costimulatory receptor (Lenschow, Walunas & Bluestone 1996). CD28 is expressed constitutively on all human CD4+ T cells and on 50% of the human CD8+ cells (June et al. 1990; Linsley & Ledbetter 1993). In contrast, all murine CD4+ and CD8+ T cells express CD28 (Gross et al. 1992). CD28's ligands are CD80 (B7-1) and CD86 (B7-2). They are both Ig superfamily members with one variable and one constant extracellular Ig-like domain (Zhang et al. 2003). CD86 is expressed at low levels on resting APCs, whereas CD80 is generally absent (McAdam et al. 1998). Both ligands are up-regulated upon activation, with differences in their kinetics. CD86 shows maximum levels of expression between 18 and 24 h after induction, whereas CD80 is not detected until 24 h post stimulation and reaches maximum levels after 48 to 72 h (Lenschow et al. 1993). Costimulatory signals mediated through CD28 facilitate T cell proliferation and trigger a number of T cell effector functions, for example cytokine secretion and protection from activation-induced cell death (see section 1.1.4., page 25).

On the molecular level, CD28 ligation along with CD3 stimulation induces activation of the phosphatidylinositol 3 (PI3) kinase (Prasad et al. 1994). This, in turn, mediates the generation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) which activates a number of enzymes, among them AKT/protein kinase B (Parry et al. 1997; Kane et al. 2001). AKT, a serine/threonine kinase, inhibits Bad, a protein involved in programmed cell death and controls numerous transcription factors, among them NF-κB (Kane et al. 1999; Ozes et al. 1999; Romashkova & Makarov 1999; Jones et al. 2000). NF-κB activation or AKT phosphorylation in turn have been observed to mediate c-FLIP$_S$ and Bcl-x$_L$ up-regulation (Boise et al. 1995; Kirchhoff et al. 2000).

1.1.1.2. CTLA-4 (CD152)

CTLA-4 (cytolytic T lymphocyte associated antigen-4) was originally identified as the fourth cDNA during a search for genes that are specifically expressed in CTLs (Brunet et al. 1987). CTLA-4 shares great sequence homology to CD28 (Harper et al. 1991)

and binds to the same ligands, but with higher affinity (Linsley et al. 1994). In contrast to CD28, CTLA-4 is not expressed constitutively on T cells. Surface expression of CTLA-4 occurs after TCR/CD28 engagement by redistribution of an intracellular pool and increased protein-synthesis (Brunner et al. 1999). Although there is data indicating that CTLA-4, unlike CD28, can be expressed as a monomer (Lindsten et al. 1993), recent publications report that CTLA-4 dimers are required for the formation of high-avidity complexes with B7 ligands and for transmission of signals that attenuate T cell activation (Ostrov et al. 2000; Schwartz et al. 2001).

CTLA-4 inhibits T cell activation by reducing IL-2 production, IL-2 receptor expression and by arresting T cells at the G_1 phase of the cell cycle (Walunas et al. 1994, Greenwald et al. 2002). Because of its higher affinity, CTLA-4 can interfere with T cell activation by sequestering B7 molecules (Carreno et al. 2000). Also, CTLA-4 can act by inducing immunosuppressive cytokine production and sequestering signalling proteins which are involved in T cell activation (Chambers et al. 2001). Costimulatory ICOS (inducible costimulator) signalling can also be inhibited by CTLA-4 (Riley et al. 2001). As mentioned above, costimulation by CD28 up-regulates CTLA-4 and is thus a self-limiting process, which supports T cell homeostasis and self tolerance. The importance of this regulating mechanism is shown in CTLA-$4^{-/-}$ mice, which die of lymphoproliferative disease within a month after birth (Tivol et al. 1995).

1.1.1.3. ICOS

The CD28 homologue ICOS (inducible costimulator) is a T cell costimulatory molecule expressed on activated T cells (Hutloff et al. 1999). Although it shares 27% and 18% sequence identity with CD28 and CTLA-4, respectively, it does not bind to CD80 or CD86. Instead, the ligand for ICOS (ICOSL, B7h) is a novel B7 family member that is constitutively expressed on B cells, macrophages and peripheral tissues (Okazaki, Iwai & Honjo 2002). Similar to CD28, signalling through ICOS can costimulate T cell proliferation, but fails to induce IL-2 production. Expression of ICOS is enhanced by CD28 costimulation and reduced when CD80 and CD86 are absent, which suggests that some functions ascribed to CD28 may be partly due to ICOS signalling (McAdam et al. 2000). Since CD28 is expressed constitutively on T cells, these differences suggest that ICOS is important for regulating activated T cells whereas CD28 functions to prime naïve T cells.

1.1.2. Costimulatory members of the tumour necrosis factor receptor family

A great variety of the the tumour necrosis factor receptor (TNFR) family members and their ligands are expressed on cells of the immune system. TNFR family members are type I integral membrane glycoproteins and defined by homology in their cysteine-rich extracellular domains (Armitage 1994). TNFR molecules can be categorised according to the presence or absence of a death domain (DD). Receptors with a DD include TNF receptor-1, CD95 and TRAIL receptor-1 among others (Armitage 1994; Pan et al. 1997). Death receptors interact via their DD with DD containing adapter molecules that link them to effector proteins (see section 1.1.3.2., page 22). Receptors lacking a DD include the nerve growth factor (NGF) receptor, TNF receptor-2, lymphotoxin (LT)β receptor, CD27, CD30, CD40, 4-1BB and OX-40 (Armitage 1994; Crowe et al. 1994; Pan et al. 1997). Their cytoplasmic tails vary largely in length and amino acid sequence, but seem to directly bind to the TNF receptor-associated factor (TRAF) family. Seven different TRAFs have been identified so far, which are defined by their carboxy-terminal TRAF domain of approximately 230 amino acids (Xu, Li & Shu 2004; Arch, Gedrich & Thompson 1998). The TRAF domain is involved in receptor interaction and in homo- and heterodimerisation of TRAFs (Takeuchi, Rothe & Goeddel 1996). TRAF-2, the best studied TRAF, can lead to NF-κB activation by the following mechanism: It binds to the NF-κB inducing kinase (NIK) which in turn activates a kinase named CHUK. CHUK then associates with and phosphorylates the NF-κB inhibitory protein IκB-α. This in turn leads to degradation of IκB-α via the ubiquitin-proteasome pathway and finally to NF-κB translocation to the nucleus (Regnier et al. 1997). Heterodimers of TRAF-1 and TRAF-2 in complex with TNFR-2 have been found to bind to cellular inhibitor of apoptosis proteins 1 and 2 (c-IAP1 and c-IAP2), which can protect against apoptosis induction (Rothe et al. 1995).

TNFR ligands share sequence identity with TNF and lymphotoxin-β, which are prototype ligands for this family (Armitage 1994). They can be expressed as soluble molecules like NGF, TNFα and LTα/TNFβ, but the majority of them are type II transmembrane proteins: CD95L, LTβ, TRAIL, CD30L, CD40L, 4-1BBL and OX-40L (Armitage 1994; Wiley et al. 1995).

1.1.2.1. 4-1BB (CD137)

4-1BB was first discovered in 1989 by screening cDNA libraries from activated murine T lymphocytes (Kwon & Weissman 1989). Its human homologue was cloned 4 years later from a library constructed from activated human T cell leukaemia virus type 1-transformed human T lymphocytes (Schwarz, Tuckwell & Lotz 1993). The human 4-1BB gene is located on chromosome 1p36 in a cluster together with the genes for OX40, Apo3, CD30 and TNFR-2 (Schwarz, Arden & Lotz 1997). It encodes for a protein consisting of 255 amino acids with two potential N-linked glycosylation sites and a molecular mass of 27 kDa. Overall, it shares 60% amino acid sequence identity to murine 4-1BB and shares five conserved regions in the cytoplasmic domain, indicating that these regions might be important for 4-1BB function (Zhou et al. 1995). The Ligand for human 4-1BB, 4-1BB Ligand (4-1BBL) was first isolated and the gene cloned in 1994. It is a type II transmembrane glycoprotein, consisting of 254 amino acids and sharing 36% identity with murine 4-1BBL (Alderson et al. 1994).
4-1BB is expressed on activated CD4+ and CD8+ T cells (Vinay & Kwon 1998) and on activated NK cells (Melero et al. 1998). Its ligand 4-1BBL is expressed on mature dendritic cells, as well as on activated B cells and macrophages (Watts & DeBenedette 1999). Unlike CD28 which is expressed constitutively, 4-1BB is induced upon primary T cell activation. Its surface expression peaks 2-3 days after stimulation (Wen, Bukczynski & Watts 2002), then declines but still remains present on activated T cells (Vinay & Kwon 1998). Signals though 4-1BB induce T cell proliferation, differentiation and protection from activation-induced cell death (Alderson et al. 1994; DeBenedette et al. 1995; Hurtado et al. 1995; Hurtado, Kim & Kwon 1997; Saoulli et al. 1998; Takahashi, Mittler & Vella 1999). Some publications conclude that costimulation via 4-1BB induces CD8 and to a lesser degree CD4 T cell proliferation (Shuford et al. 1997; Takahashi, Mittler & Vella 1999). While these studies were carried out in mice, newer studies (Cannons et al. 2001; Wen, Bukczynski & Watts 2002) indicate that CD4 and CD8 T cells are stimulated similarly by 4-1BB. Wen and coworkers, who used human T cells, report that in unfractionated T cell cultures CD4 and CD8 T cells expand to a similar extent in response to signals through the TCR and 4-1BB, while isolated CD8 T cells expand to a lesser extent than isolated CD4 T cells.

On the molecular level, it has been shown that human 4-1BB can interact with TRAF-1, -2 and -3 (Vinay & Kwon 1998), whereas murine 4-1BB can only interact with TRAF-1 and -2 (Jang et al. 1998). Of those TRAFs, TRAF-2 seems to play a central role in 4-1BB signal transduction. It has been demonstrated that a dominant negative form of TRAF-2 prevents 4-1BB-induced NF-κB up-regulation (Arch & Thompson 1998). Also, T cells from TRAF-$2^{-/-}$ mice fail to produce IL-2 upon CD3 plus 4-1BB triggering whereas they retain this ability after CD3 plus CD28 engagement (Saoulli et al. 1998). In this respect, Cannons et al. showed that 4-1BB-mediated TRAF-2 recruitment induces activation of apoptosis signal-regulating kinase 1 (ASK-1) which in turn activates the c-Jun N-terminal/stress-activated protein kinase (JNK/SAPK) pathway. Interference with the JNK/SAPK pathway by using a dominant negative form of ASK-1 prevented 4-1BB-induced IL-2 secretion (Cannons et al. 1999).

Interestingly, the transcription factors NF-κB and AP-1 are involved in TCR-dependent transcriptional regulation of the 4-1BB promotor. This regulation has been shown to be MEK- and JNK/SAPK-dependent (Kim et al. 2003).

However, while CD28 appears to play a key role during early primary T cell activation, 4-1BB-mediated stimuli seem to be more important during late primary and during secondary T cell responses (Cannons et al. 2001; Bertram et al. 2004; Dawicki & Watts 2004). In contrast to CD28-mediated signals that have been investigated to a large extent, signalling pathways involved in 4-1BB function are less well defined and therefore subject of my study.

1.1.2.2. OX40 (CD134)

The costimulatory TNFR family member OX40 is expressed on activated T cells in humans and rodents (Calderhead et al. 1993; Durkop et al. 1995). Its ligand OX40L (CD134L) is expressed on APCs after activation (Stuber et al. 1995; Ohshima et al. 1997; Murata et al. 2000), but was first described on human T lymphotropic virus-1 (HTLV-1) infected leukaemic T cells (Baum et al. 1994). Engagement of OX40 promotes effector and memory-effector T cell functions by up-regulating IL-2 production and increasing the life span of effector T cells (Weinberg, Vella & Croft 1998).

OX40 associates with TRAF-2, -3 and -5 and has been shown to activate NF-κB (Arch & Thompson 1998; Kawamata et al. 1998). While this NF-κB activation is mediated by TRAF-2 and -5, signals through TRAF-3 are likely to inhibit NF-κB activity (Kawamata et al. 1998).

In contrast to CD28, the OX40/OX40L pathway seems to be less important for the initial response of CD4 cells. T cells lacking OX40 are relatively unimpaired in IL-2 production, cell division and expansion but fail to maintain high levels of Bcl-x_L and Bcl-2 and therefore undergo apoptosis 4 - 8 days after activation (Rogers et al. 2001). OX40 expression is not dependent on CD28 signals but it has been shown that CD28 can augment OX40 expression. This supports the concept that both molecules cooperate together in a sequential manner (Rogers et al. 2001; Walker et al. 1999).

1.1.3. Apoptosis

Apoptosis, also called programmed cell death, is a key regulator of tissue homeostasis. It participates in embryonic development, differentiation, immune function and defence of pathogens. An imbalance in cell death and proliferation can lead to various diseases, for example increased apoptosis is involved in Alzheimer's disease and AIDS (Mattson 2000; Rathmell & Thompson 2002), whereas impaired apoptosis can lead to cancer or autoimmune disease (Bakhshi et al. 1985; Fisher et al. 1995).

Apoptosis is an evolutionarily conserved process, which is found in organisms as different as *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens* (Bergmann, Agapite & Steller 1998). The term apoptosis was introduced 1972 by Kerr, Wyllie and Currie to describe a form of cell death distinct from necrosis (Kerr, Wyllie and Currie 1972). Necrosis is a passive, accidental form of cell death, caused by unphysiological changes in the cellular environment or toxic agents. It is characterised by swelling of the cell and membrane disruption, evoking an inflammatory reaction in the neighbouring cells. In contrast, during apoptosis cells undergo well organised morphological and biochemical changes, such as cell shrinkage, membrane blebbing, nuclear DNA fragmentation and exposure of phosphatidylserine from the inner to the outer membrane leaflet (Hengartner 2000). This change in membrane surface triggers the recognition and removal of the apoptotic cell by macrophages and other phagocytes, causing no inflammation (Fadok et al. 2001).

1.1.3.1. Caspases

Apoptosis is arranged by the action of a set of cellular proteases, called caspases (cysteine proteinases with specificity for aspartate residues). Activated caspases cleave substrates, either activating or inactivating them. Cleavage of these substrates produce the observed morphological and biochemical changes and provide markers for the quantification of apoptotic death. Cleavage occurs only at the carboxyl side of aspartate residues (Earnshaw, Martins & Kaufmann 1999). Caspases can only be cleaved and thereby activated by themselves or by a serine protease called granzyme B. This enzyme is present in the granules of cytotoxic lymphocytes and transferred to target cells in the killing process. By directly cleaving caspases, granzyme B can bypass many sites of regulation that may be exploited by intracellular parasites (Greenberg 1996, Pham & Ley 1997; Thornberry et al. 1997).

Caspases are synthesised as zymogens with an N-terminal prodomain, followed by a large (~20 kDa) and a small (~10 kDa) subunit. The length of the prodomain varies within different caspases; Initiator caspases (caspases 2, 8, 9 and 10 in vertebrates) possess long prodomains that undergo autoproteolytic activation when aggregated by cytoplasmic adapter molecules. This interaction is mediated by the death effector domain (DED) or caspase recruitment domain (CARD) in the caspase prodomain. For example, caspase 8 and 10 have two DEDs and one of them interacts with the DED in FADD (Fas associated death domain) (Cohen 1997). Similarly, the CARD of caspase 9 binds to that of Apaf-1 (Hu et al. 1998). Autocatalytic cleavage removes the prodomain and yields the small and the large subunits that are assembled to the active form of the caspase (Hengartner 2000). Subsequently, these activated initiator caspases can cleave and activate effector caspases with short prodomains (caspases 3, 6 and 7 in vertebrates). These in turn cleave various substrates, among them structural proteins and proteins involved in DNA repair and cell cycle regulation (Cohen 1997). These substrates explain the observed phenomena during apoptosis; for example, CAD (caspase-activated DNAse) is responsible for the nuclear DNA fragmentation (Sakahira, Enari & Nagata 1998), whereas cleavage of the cytoskeletal protein gelsolin may account for the loss of overall cell shape (Kothakota 1997).

1.1.3.2. Extrinsic death pathway

Death receptor function is typified by the TNF receptor family member CD95 (Apo-1/Fas) (Nagata & Golstein 1995) (see also chapter 1.1.2., page 17). Death receptors contain an intracellular death domain (DD) which is essential for transduction of the death signal. Trimerisation of CD95 by its natural ligand CD95L leads to formation of the death inducing signal complex (DISC) (Fig. 3). Complex formation starts with the recruitment of the adapter protein FADD with its DD (Krammer 1999). The opposite side of FADD contains two DEDs that recruit caspase 8 (FLICE, Fas-associated death-domain-like IL-1β-converting enzyme) to the DISC, in which the caspase becomes activated and in turn can activate effector caspases (Muzio et al. 1996) (see section 1.1.3.1., page 21). The caspase 8 homologue c-FLIP (cellular FLICE inhibitory protein) is an important inhibitor of the extrinsic pathway of apoptosis. At the protein level, c-FLIP exists in two different splice variants, the 26 kDa c-$FLIP_{Short}$ (c-$FLIP_S$) and the 55 kDa c-$FLIP_{Long}$ (c-$FLIP_L$) (Fig. 2). c-$FLIP_L$ contains two DEDs and a caspase-like domain, which lacks proteolytic activity. c-$FLIP_S$ consists only of two DEDs

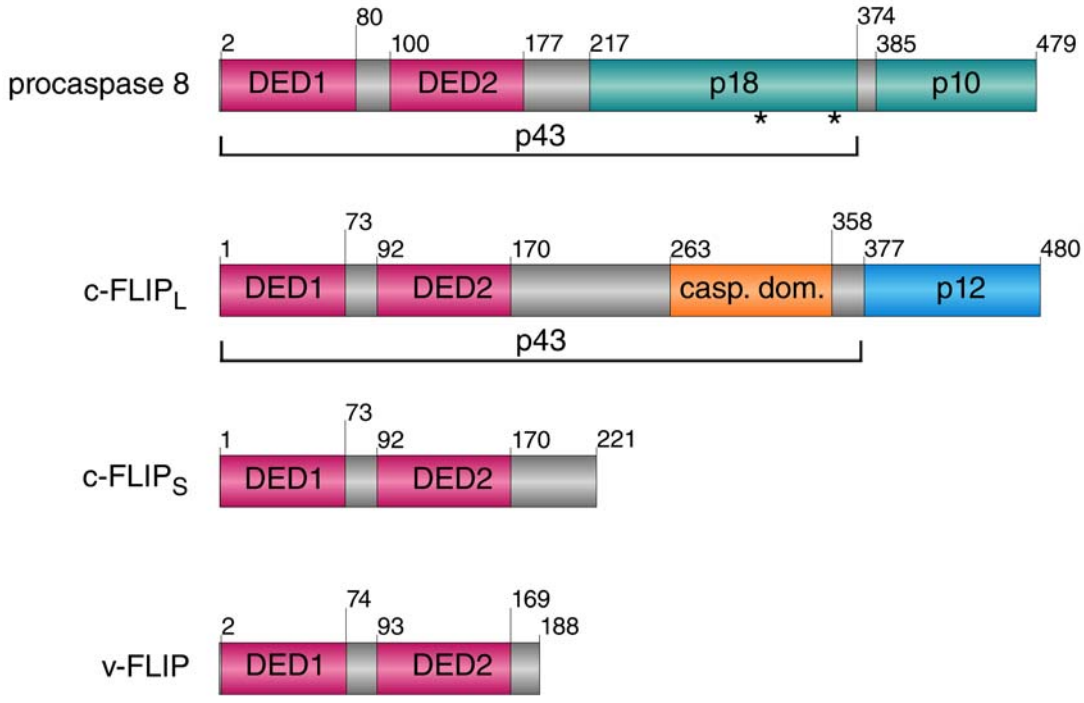

Fig. 2: Structural similarities between caspase 8 and FLIP. *Both procaspase 8 and the various forms of FLIP contain two repeats of death effector domains. c-$FLIP_S$ and v-FLIP have only short c-terminal extensions, whereas c-$FLIP_L$ contains a long c-terminal extension which is homologous to caspase 8 but lacks proteolytic activity. The active sites of caspase 8 are indicated by stars. Amino acid numbers and domains are according to the Swiss-Prot sequences of human procaspase 8 (Q14790), human c-$FLIP_L$ (O15519) and human c-$FLIP_S$ (O15519-2) or according to the TrEMBL sequence for v-FLIP of human herpesvirus 8 (P88961).*

and a short C-terminal part. Both forms of c-FLIP are recruited to the DISC via their DEDs but they differ in their mode of inhibition. c-FLIP$_L$ and caspase 8 form a heterodimer and are both partially processed to inactive fragments of 10/12 and 43 kDa in size (Kataoka & Tschopp 2004). In contrast, binding of c-FLIP$_S$ to the DISC completely abolishes caspase processing (Thome & Tschopp 2001). Structurally similar to c-FLIP$_S$, some viruses express viral FLIP proteins (v-FLIP) which inhibit death receptor signalling in the same manner (Fig. 2). These v-FLIPs are important for viral reproduction, as they impede killing of the infected cell by the hosts immune system. Also, they contribute to the oncogenity of some herpesviruses (Thome et al. 1997; Bertin et al. 1997).

1.1.3.3. Intrinsic death pathway

While the events leading to activation of the extrinsic pathway involve ligation with a death inducing ligand, for example CD95L or TRAIL, the intrinsic death pathway is activated by various death stimuli, such as growth factor deprivation, chemotherapeutic drugs, ionising radiation and DNA damage. These stimuli mediate Bax or Bak activation, which subsequently mediates loss of mitochondrial transmembrane potential (Fig. 3). This coincides with the release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol. Cytosolic cytochrome *c* binds to Apaf-1, a cytosolic protein cofactor containing a CARD (Zou et al. 1999). This binding increases the affinity of Apaf-1 to ATP, which in turn triggers the formation of the oligomeric apoptosome. Caspase 9, the initiator caspase of the intrinsic death pathway, is recruited via its CARD to the apoptosome and becomes activated. Unlike other caspases, this activation is not mediated by proteolytic processing, but by its association with the protein cofactor Apaf-1 (Rodriguez & Lazebnik 1999; Stennicke et al. 1999). As mentioned above, the intrinsic death pathway is triggered by the release of cytochrome *c*. It is not exactly known how cytochrome *c* is translocated through the mitochondrial outer membrane, but there is evidence that bcl-2 family members are involved in this process. Bax and Bak, pro-apoptotic members of the bcl-2 family, have been found to allow cytochrome *c* to pass through the voltage-dependent anion channel (VDAC) out of liposomes, whereas the anti-apoptotic bcl-2 family member Bcl-x$_L$ inhibits cytochrome *c* release (Daniel et al. 2003). Bid, another member of the bcl-2 family,

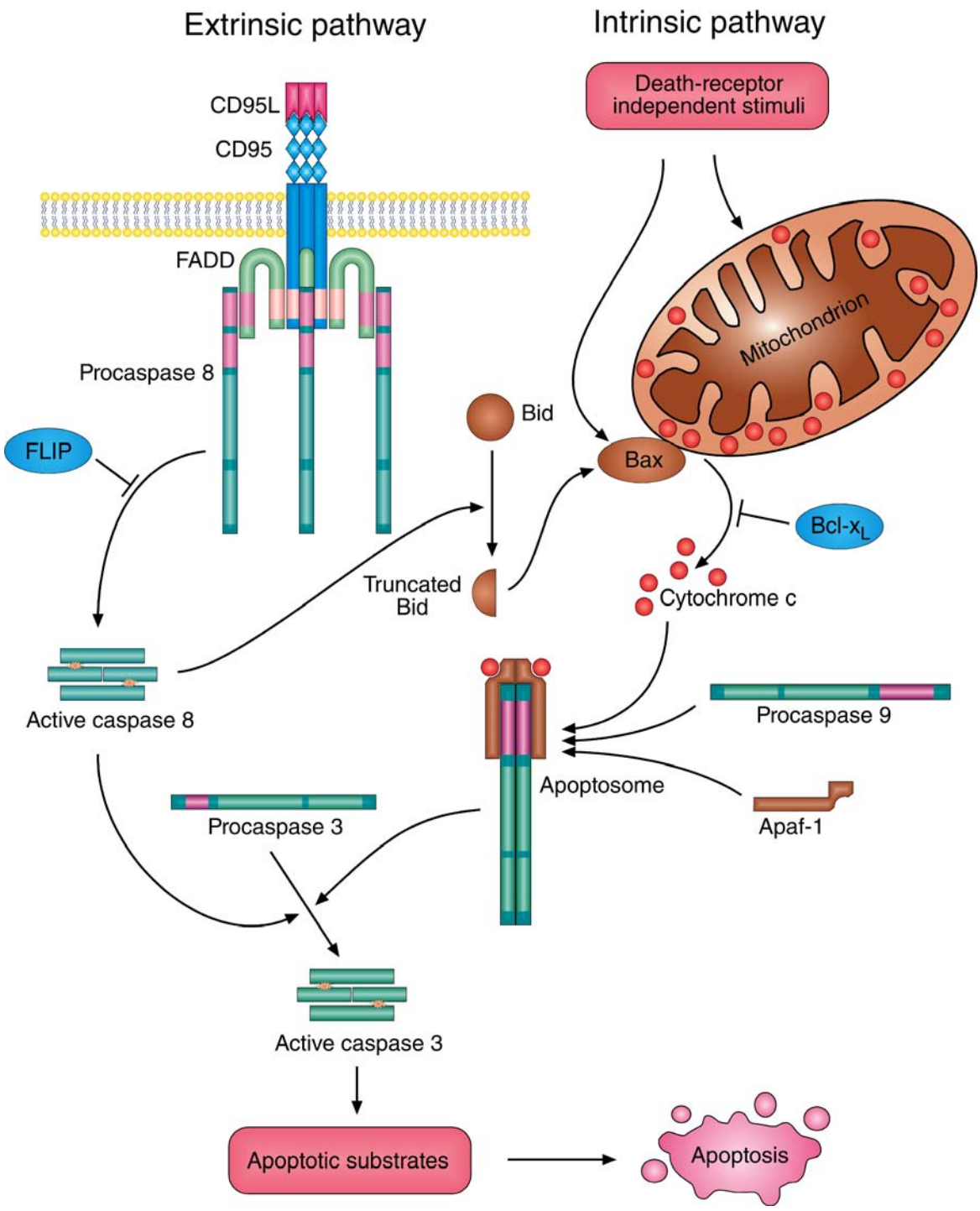

Fig. 3: Two pathways of apoptosis. *The extrinsic death pathway is triggered by death receptors like CD95. Binding of CD95L to CD95 leads to receptor trimerisation and formation of a death inducing signal complex, consisting of CD95L, the CD95 receptor, the adapter protein FADD and procaspase 8. Caspase 8 is activated in this complex and in turn activates the effector-caspase 3. FLIP interferes with the extrinsic pathway by inhibiting caspase 8 activation (see text).*
The intrinsic death pathway is induced by various stimuli, such as DNA damage, growth factor deprivation, irradiation and chemotherapeutic drugs, leading to loss of the mitochondrial transmembrane potential and release of cytochrome c into the cytosol. Cytochrome c, together with Apaf-1 and procaspase 9 form the mitochondrial apoptosome, which leads to activation of caspase 9 and subsequently to activation of caspase 3. Bcl-x_L interferes with the intrinsic death pathway by inhibiting cytochrome c release. Caspase 8 mediated cleavage of Bid promotes cytochrome c release and thus connects the extrinsic to the intrinsic apoptosis pathway (see text).

can promote cytochrome *c* release from the mitochondria when cleaved by caspase 8 (Gross et al. 1999). This indicates the existence of a crosstalk between the extrinsic and intrinsic death pathway (Daniel 2000).

1.1.4. Activation-induced cell death

Lymphocyte homeostasis is a tightly regulated process that often involves cell death. Previous results demonstrated that TCR ligation in the absence of costimulation induces anergy during primary T cell activation and mediates activation-induced cell death (AICD) during restimulation (Daniel et al. 1997a, 1998, 1999; Scholz et al. 2002). Elimination of T cells by AICD serves as an important mechanism to terminate immune responses and mainly occurs via CD95 through the extrinsic pathway of apoptosis (Alderson et al. 1995). Apart from the extrinsic pathway of apoptosis, the mitochondrial or intrinsic pathway of apoptosis mediates cell death, for example in response to growth factor deprivation. These pathways are interconnected as caspase 8 and 3 mediate cleavage of the pro-apoptotic Bcl-2 family member Bid which in turn triggers Bax or Bak activation and subsequently cytochrome *c* release and apoptosome formation (see section 1.1.3.3., page 23). Interference with AICD and survival of T cells is mediated by induction of Bcl-x_L (Schmitz et al. 2003), an anti-apoptotic Bcl-2 family member that hampers the intrinsic pathway of apoptosis on the mitochondrial level, while c-FLIP directly inhibits caspase 8 activity (Fig. 3) (Bertin et al. 1997; Hu et al. 1997; Thome et al. 1997). In T cells, c-$FLIP_S$ expression can be induced upon ligation of the costimulatory molecule and immunoglobulin super-family member CD28 (Kirchhoff et al. 2000).

1.2. Tumour immunology

In the year 1909 the immunologist Paul Ehrlich formulated the idea that tumours arise continuously in our bodies and are removed by the immune system before they become clinically manifested (Ehrlich 1909). Indeed, there is experimental evidence that the immune system can eradicate tumours. Early tumour transplantation models suggested the existence of tumour-associated antigens (Gross 1943; Prehn & Main 1957; Klein et al. 1960) and led to the “immunosurveillance theory”, postulated by Sir Macfarlane Burnet and Lewis Thomas (Burnet 1957, 1970; Thomas 1959). Burnet hypothesises that antigenic changes of incipient tumours provoke an effective immu-

nological reaction with regression of the tumour and thereby leaving no clinical hint of its existence. The postulated absence of traces makes the immunosurveillance theory hard to prove and is thus controversially discussed (Dunn et al. 2002; Dickey 2002; Qin & Blankenstein 2004). For example, it has been reported that immunosuppressed organ allograft recipients have a 3- to 4-fold increased risk of developing tumours and furthermore the incidence of certain, usually rare cancers like post-transplant lymphoproliferative disorders (PTLD) and Kaposi's sarcoma (KS) is increased more than a hundredfold. This would support the immunosurveillance theory, but a closer look reveals a viral aetiology in most of these tumours (Penn 2000). It is undoubted that the immune system has a protective function against viral pathogens and thus can eliminate virally induced tumours (Rickinson & Moss 1997; Flaitz & Hicks 1998; Mueller 1999; Callan 2004). Reports of spontaneous tumour regression are rare and a viral tumour aetiology can not be excluded in all these cases (Chang et al. 2004; Cui & Willingham 2004; Oya & Ikemura 2004; Fritsch et al. 2004). Supporting the immunosurveillance theory, NK cells have been shown to kill MHC class I-deficient tumour cells and their metastases (Whiteside & Herberman 1995).

Interestingly, in paraneoplastic neurologic degenerations (PNDs) there is a link between tumour immunity and autoimmune neurologic disease: Tumours from PND patients ectopically express neuronal antigens and patients harbour high-titer antibodies in their blood and spinal fluids directed against these proteins. The neurologic symptoms lead to early diagnosis of the tumour, and cytolytic T cells directed against these neuronal antigens are found in the peripheral blood of PND patients. Also, these patients have clinical and pathological evidence of suppression of tumour growth. Nevertheless, spontaneous tumour regression in PND patients is also a rare event (Darnell & Posner 2003).

Instead of fighting cancer, inappropriate immune reactions can also promote tumour development. Chronic inflammation, probably an attempt of the host to suppress tumour growth, can produce an attractive environment for tumours (Coussens & Werb 2002). Tumour infiltrating leukocytes for example induce DNA damage in proliferating cells through their generation of reactive oxygen and nitrogen species. These species react to form peroxynitrate, a cytotoxic and mutagenic agent and are normally produced by leukocytes to fight infections (Maeda & Akaike 1998).

Macrophage infiltration is observed in a range of tumour types. Tumour associated macrophages (TAMs) are derived from monocytes that are recruited to the tumour by tumour cell expressed chemoattractant cytokines. Within the tumour microenvironment, monocytes differentiate into macrophages (Tang et al. 1992; Lewis et al. 2000). Usually, macrophages are involved in antigen presentation, target cell cytotoxicity, removal of debris and tissue remodelling, regulation of inflammation and induction of immunity. In contrast, TAMs mostly do not exert cytotoxicity but rather promote tumour growth, vascularisation and metastasis. Tumours secrete cytokines like IL-4, IL-10, transforming growth factor-β (TGF-β), prostaglandin E-2 (PGE-2), macrophage colony stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF), which modulate the cytotoxic phenotype of macrophages into a suppressive phenotype. Moreover, there is evidence that TAMs can suppress T cell function by the production of prostaglandins and immunosuppressive cytokines such as TGF-β and IL-10. Altered antigen presentation by TAMs is also discussed as a mechanism of suppressing anti-tumour responses (al-Sarireh & Eremin 2000). In breast cancer, TAMs were shown to produce several angiogenic factors that stimulate tumour neovasculature. Also, there was a correlation between macrophage infiltration, vasculation grade and reduced relapse-free or overall survival (Leek et al. 1996). Not only macrophages, but also other immune cells like neutrophils, mast cells, eosinophils and activated T lymphocytes can contribute to malignancies by releasing extracellular proteases, pro-angiogenic factors and chemokines (Coussens & Werb 2002).

1.2.1. Cancer vaccines

Vaccines against infectious agents prepare the immune system to encounter with pathogens by establishing a humoral immunity. This strategy is successful when pathogens possess defined antigens that are different from self. Tumours however, possess an unlimited number of potential antigens that arise during tumourigenesis. These antigens are either not expressed or expressed at low levels by normal tissues. The former include viral gene products, products of chromosomal translocations and point mutations, the latter include transcription factors, differentiation antigens and reactivated embryonic gene products (Greten & Jaffee 1999; Boon & van der Bruggen 1996).
Unlike prophylactic vaccines for infectious agents, therapeutic cancer vaccines attempt to activate immune responses against antigens which the immune system has already been exposed to. Unfortunately, the outcome of this first contact with antigens

that arise during tumourigenesis is tolerance rather than activation (Staveley-O'Carroll et al. 1998; Speiser et al. 1997). Tumours have evolved various mechanisms to evade the immune system. Generation of antigen-loss tumour variants, loss of MHC expression, down-regulation of the antigen processing machinery and expression of inhibitory molecules like TNF-β and Fas ligand are examples of acquired resistance that can play a role in some cancers (Pardoll 1998).

The context in which the antigen is presented to the immune system determines the outcome of an immune reaction. Successful T cell activation requires the presence of costimulatory signals at the time of antigen recognition (see section 1.1., page 13).

Tumours are poor stimulators of immune responses and can induce T cell anergy in murine models (Staveley-O'Carroll et al. 1998). Therefore, to be effective, cancer vaccines must either break tolerance or activate a T cell population that escaped tolerance because of their low affinity for antigens expressed by the tumour (Nanda & Sercarz 1995; Chen 1998). Recent findings have established the propensity of cytokines and costimulatory signals to generate T cell responses in clinical and preclinical vaccination studies (Cayeux et al. 1995; Blankenstein, Cayeux & Qin 1996; Cayeux et al. 1996). Moreover, there is evidence that tumour cells are capable of directly and indirectly activating tumour-specific T lymphocytes (Huang et al. 1996). Indirect activation or cross-priming involves tumour antigens presented to T cells in the context of MHC class I or II on antigen presenting cells (APC), for example dendritic cells (DC). In contrast, direct T cell activation is facilitated as tumour cells frequently express MHC class I and thus are capable of directly presenting tumour antigens to cytotoxic T lymphocytes (CTL). Direct T cell activation is largely improved when tumour cells are genetically engineered to express costimulatory ligands (Fig. 1), for example CD80 (Cayeux et al. 1995; Cayeux et al. 1996; Huang et al. 1996). Adenoviral vectors encoding IL-7/CD80 are successfully employed to efficiently engineer established tumours *in vivo* (Willimsky & Blankenstein 2000) and to generate gene-modified *ex vivo* tumour cell vaccines (Scholz et al. 2002). Alternatively, T cell targeting and activation against tumour cells can be achieved by bispecific antibodies in conjunction with costimulation by antibodies against, for example, CD28 on T cells (Daniel et al. 1998).

Similar to preclinical murine models, human autologous tumour cells or allogeneic tumour cell lines have been employed as vaccines in clinical trials (Greten & Jaffee 1999; Simons et al. 1999; Vermorken et al. 1999; Nawrocki et al. 2000; Kusumoto et al. 2001; Jocham et al. 2004). Such tumour cell vaccines appear to be interesting in adjuvant settings, i.e. where the main tumour load has been removed and putatively only minimal residual disease (MRD) remains. For fear of inducing new tumour burden, tumour cell vaccines in humans are generally inactivated before inoculation. Accordingly, tumour cells were typically irradiated to abrogate clonogenic growth in a number of clinical studies (Harms-Ringdahl, Nicotera & Radford 1996; Shinomiya 2001; Zhou, Yuan & Serggio 2003). Alternatively, tumour cell vaccines are inactivated by repeated freeze and thaw cycles. While the latter method renders tumour cells necrotic, cell irradiation is believed to primarily induce apoptosis. There is conflicting data in the literature as to whether vaccine inactivation abrogates its capacity to modulate the immune system. In this context, data from murine tumour models demonstrated that irradiation of the tumour cell vaccine diminishes its anti-tumour effect (Hock et al. 1993a; Cayeux et al. 1995, 1996). Furthermore it is not clear whether necrotic cells as compared to apoptotic tumour cells differ in regard to their capacity to directly modulate the immune system. A thorough understanding of the immunomodulating capacity of tumour cell vaccines, however, is of great importance for the design of clinical vaccine trials.