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Inhibitors of apoptosis confer resistance to tumour suppression by adoptively transplanted cytotoxic T-lymphocytes *in vitro* and *in vivo*

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Abstract

Deregulation of apoptosis signalling is commonly found in cancer and results in resistance to cytotoxic therapies. Immunotherapy is a promising strategy to eliminate resistant cancer cells. The transfer of T-lymphocytes during allogeneic stem cell transplantation is clinically explored to induce a 'graft-versus-tumor' effect (GvT). Cytotoxic T-lymphocytes (CTL), which are major effectors of GvT, eliminate cancer cells by inducing apoptosis via multiple parallel pathways. Here, we study *in vitro* and *in vivo* the susceptibility of murine cancer cells engineered to express single antiapoptotic genes to CTL-mediated cytotoxicity. Interestingly, we find that single inhibitors of caspase activation, such as BCL-XL or dominant-negative mutants of FADD and caspase-9, protect cancer cells against antigen-specific CTL *in vitro*. Moreover, expression of BCL-XL impairs the growth suppression by adoptively transplanted CTL of established tumours *in vivo*. Hence, apoptosis defects that provide protection to cytotoxic cancer therapies can confer crossresistance to immunotherapy by tumour-reactive CTL.
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Keywords: transplantation; cancer immunology; resistance; cytotoxicity

Abbreviations: ASCT, allogeneic stem cell transplantation; DISC, death-inducing signalling complex; MEF, murine embryo fibroblasts; MOMP, mitochondrial outer membrane permeabilization; PI, propidium iodide; TAA, tumour-associated antigen

Introduction

Genetic deregulation of apoptosis signalling is a frequent event in malignant transformation and tumour progression.[1] Oncogenes, such as Myc and Ras, trigger p53-dependent apoptosis and senescence via the gene products of the INK4A locus.[2,3] Accordingly, genetic alterations inactivating the ARF/p53/RB pathway are strongly selected during oncogenesis.[4,5] In addition, impaired apoptotic signalling via the endogenous ('mitochondrial') pathway of caspase activation downstream of p53 provides further selective advantage in several cancer models.[6–8] Hence, most established cancers harbour inherent defects in this apoptotic signalling cascade. Interestingly, clinically applied cytotoxic therapies, such as γ-radiation and most anticancer drugs, also induce apoptosis via p53 and the 'mitochondrial' apoptotic pathway.[9,10] As a consequence, apoptosis defects selected during oncogenesis and tumour progression can simultaneously confer resistance to anticancer therapy.[11]

Taking this into consideration, rationally designed cancer treatments should be able to bypass such genetic blocks in the transduction of apoptotic death signals. Cellular immune effectors, such as tumour-specific cytotoxic T-lymphocytes (CTLs), are thought to meet this requirement, and combine it with their capability to induce target cell apoptosis in a highly selective manner. Mechanistically, CTL induce caspase activation and apoptosis via at least two parallel pathways: (a) death receptors, such as CD95/Fas/APO-1 expressed on tumour cells, interact with their respective ligands expressed by CTL to trigger caspase activation through the formation of the death-inducing signalling complex (DISC).[12] (b) Cytotoxic granules from CTL contain perforin and granzyme B, which cooperatively activate caspases of the cancer cells.[13,14] In addition, alternative death pathways mediated by granzyme A have recently been characterized, which also feed into CTL-induced apoptosis.[15,16]

Clinically, the therapeutic potential of CTL is broadly explored in the context of allogeneic stem cell transplantation (ASCT) protocols for resistant haematopoietic and nonhaematopoietic cancers. Despite impressive clinical successes, recurrent disease still is a major cause of mortality following ASCT immunotherapy, and thus calls for further improvement of this therapeutic modality. To dissect systematically the relative contribution of the various CTL-induced pathways of apoptotic caspase activation to cancer cell elimination, we studied *in vitro* and *in vivo* the susceptibility of murine tumours engineered to express antiapoptotic genes to the cytotoxic effects of alloreactive CTL and CTL specific for a tumour-associated antigen (TAA). Despite the presence of parallel proapoptotic effector mechanisms, we found that the expression of single genetic inhibitors of caspase activation, which predominantly inhibit one pathway, can impair the tumour-suppressive activity of antigen-specific CTL.

Results

Generation and characterization of transgenic murine cancer cells

To obtain genetically defined cancer cells, we generated murine embryo fibroblasts (MEF) from $p53^{-/-}$ mice backcrossed onto a human leucocyte antigen (HLA)-$A2K^b$ transgenic background ($p53^{-/-}$ $A2K^b$). These MEF were sequentially transduced with retroviral vectors expressing the oncogenes *E1A* and H-*ras*, and the human mutant $p53^{V143A}$ cDNA as tumour-associated target for CTL. In coculture experiments, allo-$A2K^b$-reactive murine $CD8^+$ CTL (CD8 allo-A2) specifically lysed such $A2K^b$-transgenic, p53-reconstituted MEF and reduced their proliferative survival (Figure 1a). The cytotoxicity of allo-$A2K^b$-reactive CTL required cellular contact with the MEF targets (Figure 1b), and was abolished by treatment with ethylenediaminetetraacetic acid (EDTA) and magnesium chloride (Figure 1c). These results ruled out a role for secreted factors in our experimental system, and pointed towards an effector mechanism predominantly involving the granule-dependent pathway.[17]

To study the relative contribution of key steps of the caspase activation cascades in CTL-induced cytotoxicity, we expressed a set of apoptosis inhibitors in these MEF cancer cells (Suppl. Figure 1a): This included BCL-XL, which counteracts the mitochondrial outer membrane permeabilization (MOMP) through proapoptotic BCL-2 family proteins,[18] a catalytically inactive mutant caspase-9^{C287A} (DN-Casp-9), which is thought to prevent APAF-1-dependent caspase activation,[19] a truncated FADD protein (DN-FADD), which interferes with death receptor-induced caspase activation,[20] and a truncated X-linked inhibitor of apoptosis (XIAP) protein ($XIAP^{\Delta RING}$), which is resistant to proteasomal degradation and inhibits activated caspases.[21] In order to avoid selection phenomena and to discriminate MEF cancer cells from cocultured CTL, bicistronic retroviral vectors were employed, which expressed the respective apoptosis inhibitor and green fluorescent protein (GFP). Successfully transduced MEF populations were obtained by fluorescence-activated cell sorting.

To confirm biologically relevant expression levels of the apoptosis inhibitors, we treated the respective MEF populations

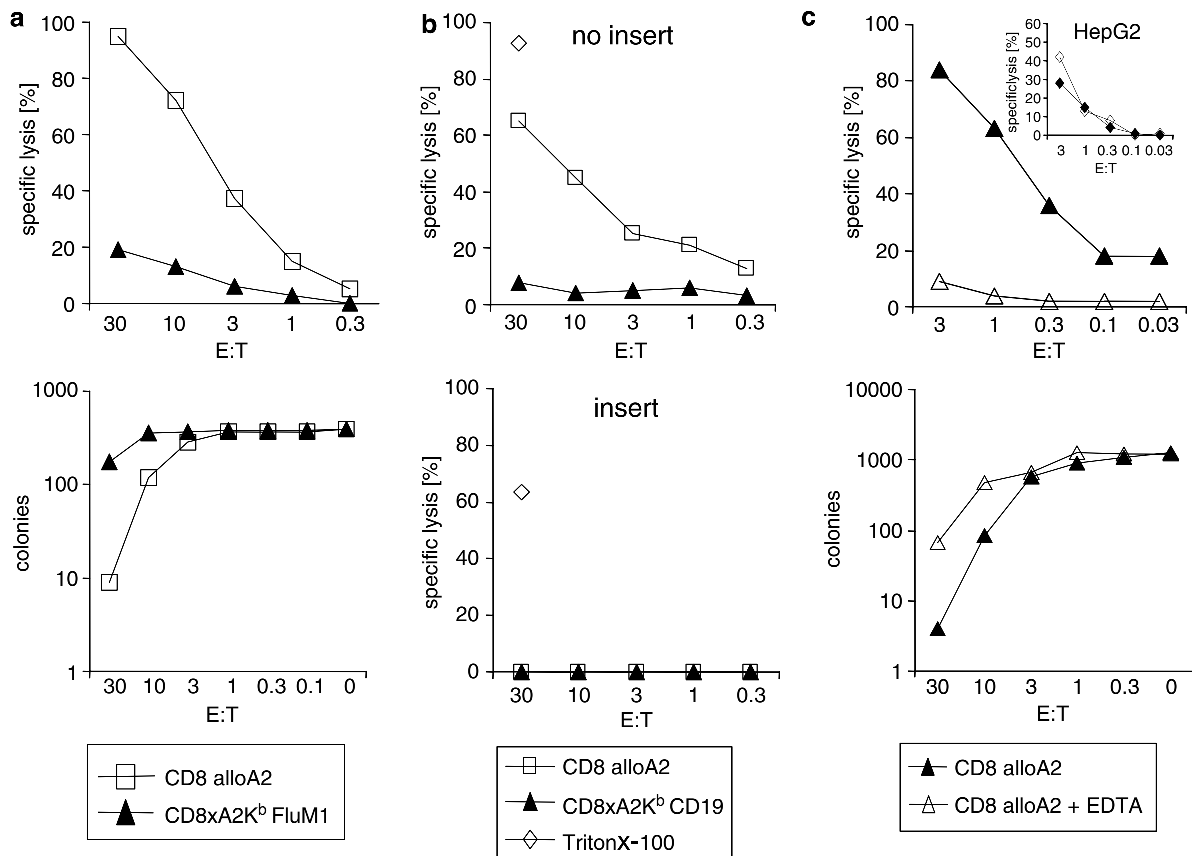

Figure 1 Antigen-specific, contact-dependent lysis of $p53^{-/-}$ $A2K^b$ MEF cancer cells by CTL via the granule-mediated pathway. (**a**) The 5 h ^{51}Cr release assay (upper panel) and proliferative survival (lower panel) of $p53^{-/-}$ $A2K^b$ MEF coincubated with allo-$A2K^b$-reactive (CD8 allo-A2, open boxes) or control CTL (CD8 $\times$ $A2K^b$ FluM1, closed triangles). (**b**) The 5 h ^{51}Cr release assay of $p53^{-/-}$ $A2K^b$ MEF coincubated with allo-$A2K^b$-reactive (CD8 allo-A2, open boxes) or control CTL (CD8 $\times$ $A2K^b$ CD19, closed triangles) in the absence (upper panel) or presence (lower panel) of separating membrane inserts. Triton X-100 (open diamonds) indicates the maximum ^{51}Cr release as achieved by incubation with membrane-permeable detergent. (**c**) The 5 h ^{51}Cr release assay (upper panel) and proliferative survival (lower panel) of $p53^{-/-}$ $A2K^b$ MEF coincubated with allo-$A2K^b$-reactive (CD8 allo-A2) CTL in the absence (closed triangles) or presence (open triangles) of 4 mM EDTA and 2 mM $MgCl_2$. The insert demonstrates that the predominantly CD95-dependent lysis of HepG2 targets by allo-$A2K^b$-reactive CTL is not inhibited by EDTA/$MgCl_2$. Mean values of duplicates of one of at least three independent experiments are shown

with cytotoxic drugs, UV radiation and TNF (Supplemental Figure 1b). As expected, expression of BCL-XL, DN-Casp-9 or XIAP$^{\Delta RING}$ conferred protection against apoptosis induced by cytotoxic drugs and UV radiation, whereas DN-FADD significantly reduced apoptosis in MEF treated with TNF plus cycloheximide.

Inhibitors of caspase activation protect cancer cells against CTL lysis *in vitro*

To study the activity of our genetic inhibitors of apoptosis against CTL-mediated cytotoxicity, we devised two CTL populations with the following specificities: allo-A2K^b-reactive CTL lyse targets that express the HLA-A*0201 antigen, and A2 p53.264 CTL that lyse targets presenting the human p53 (264–272) epitope in the context of HLA-A*0201.[22,23] The expression of BCL-XL, but none of the other apoptosis inhibitors, significantly protected MEF against cytolysis in coculture experiments with allo-A2K^b-reactive CTL. This protection translated into a two- to three-fold increase in proliferative survival of BCL-XL-expressing MEF *in vitro* (Figure 2). When studying the human mutant p53^{V143A} as CTL target, a different picture emerged. Whereas XIAP$^{\Delta RING}$ expression conferred no protection against HLA-A*0201-restricted CTL specific for the human p53 (264–272) epitope (A2 p53.264), DN-FADD and DN-Casp-9 expression significantly reduced cytolysis *in vitro*. Again, BCL-XL expression resulted in the strongest protection of cancer cells against p53-specific CTL lysis. Moreover, BCL-XL increased the proliferative survival of MEF cocultured with p53-specific CTL approximately 10-fold, whereas DN-FADD or DN-Casp-9 failed to do so (Figure 2). In summary, expression of BCL-XL conferred the strongest protection against the cytotoxic effects of two different, antigen-specific CTL populations.

BCL-XL protects against CTL-mediated cytotoxicity by preventing mitochondrial damage and caspase activation

BCL-XL is thought to prevent caspase activation and apoptosis by sequestering proapoptotic BH3 proteins, such as BIM or BID.[24] Recently, it was shown that apoptosis induced by recombinant granzyme B, a major effector of CTL-mediated cell death, may involve an activating cleavage of BID to induce MOMP and apoptosis, and hence can be blocked by BCL-2 or BCL-XL.[25–27] However, conflicting observations on the protection by BCL-2 against apoptosis induced by natural T cells, which harbour additional cytotoxic effectors besides granzyme B, have been reported.[28–31] To confirm that the BCL-XL-mediated protection in our

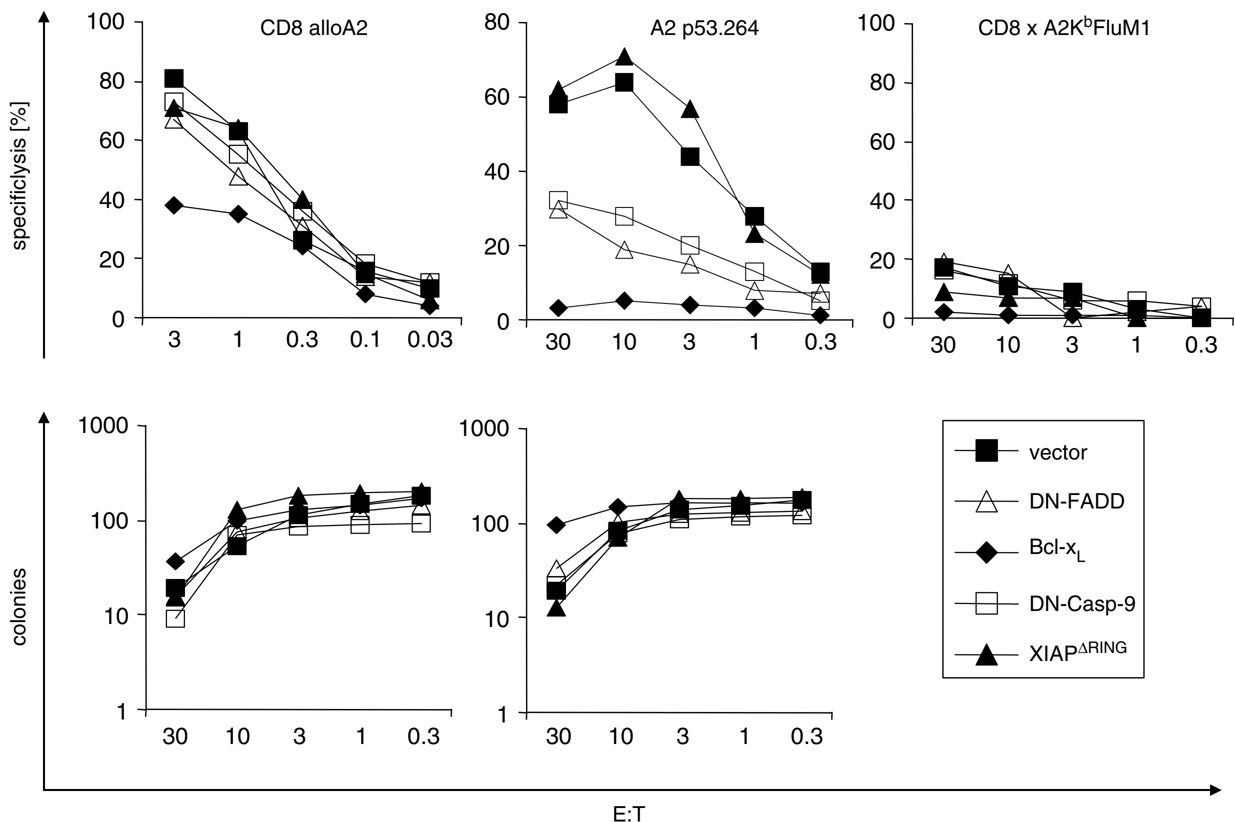

Figure 2 Expression of apoptosis inhibitors protects cancer cells against CTL-induced cytotoxicity *in vitro*. CTL-induced specific lysis of MEF cancer cells expressing DN-FADD (open triangles), BCL-XL (closed diamonds), DN-Casp-9 (open boxes), XIAP$^{\Delta RING}$ (closed triangles) or control vector (closed boxes) in representative 5 h ^{51}Cr release assays (upper panel). Mean colony formation (logarithmic scale) of MEF cancer cells after coincubation with CTL (lower panel). The specificities of the respective CTL are indicated; CD8 $\times$ A2K^b FluM1 served as negative control CTL. Mean values of duplicates of at least three independent experiments are shown

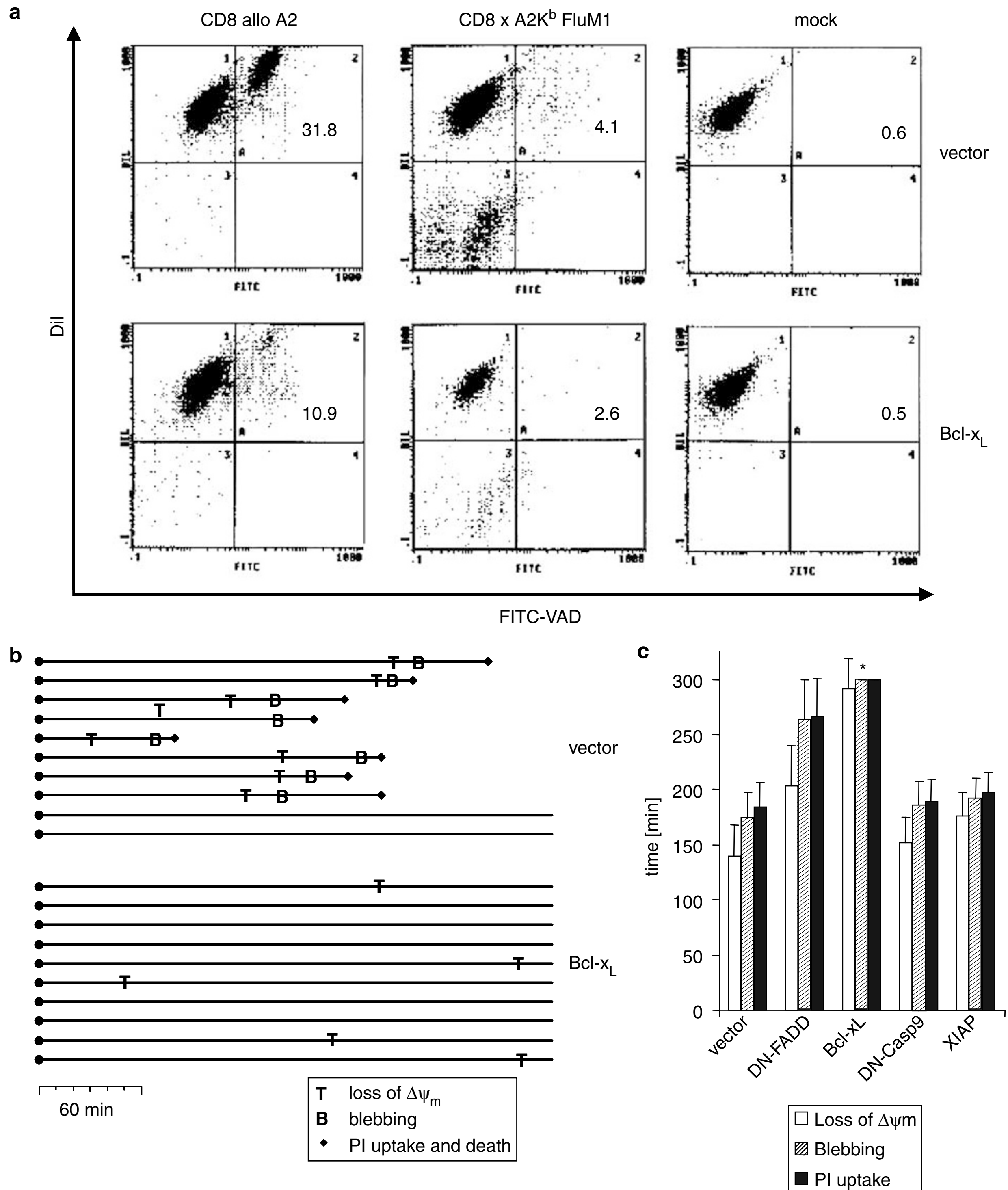

Figure 3 BCL-XL prevents CTL-induced caspase activation, mitochondrial damage and apoptosis. (**a**) MEF cancer cells expressing BCL-XL or control vector were loaded with the fluorescent dye DiI and coincubated with allo-A2K^b-reactive CTL (CD8 allo-A2) at an E : T of 0.1; effector caspase activity was detected by staining with FITC-VAD. The numbers indicate the fraction of DiI$^+$/FITC$^+$ MEF of one representative of four independent experiments. Influenza matrix peptide-specific CTL (CD8 $\times$ A2K^b FluM1, E : T of 1) served as negative control. (**b**) Timing of apoptotic events in a representative time lapse of 10 individual MEF cancer cells selected from one field expressing BCL-XL or control vector following coincubation with p53-specific CTL (A2 p53.149). 'T' denotes loss of $\Delta\psi_m$ (indicated by the loss of TMRE staining), 'B' denotes membrane blebbing of GFP-positive cells and a closed diamond indicates plasma membrane permeabilization (indicated by PI uptake), which was quickly followed by rounding up and detachment of the cells. (**c**) Mean time (+ S.E.) of the onset of loss of $\Delta\psi_m$ (open bars), membrane blebbing (hatched bars) and PI uptake (closed bars) in representative time lapses of at least 10 MEF cancer cells per field expressing the indicated apoptosis inhibitors that were coincubated with p53-specific CTL. The asterisk indicates that blebbing and PI uptake were not observed during the assay time of 5 h in BCL-XL-expressing MEF

experimental system results from the prevention of MOMP and caspase activation, we studied the apoptosis of MEF incubated with CTL in two different assays at a single-cell level. The expression of BCL-XL profoundly inhibited caspase activation in MEF cocultured with allo-A2K^b-reactive CTL (Figure 3a). This was accompanied by a delay in CTL-induced mitochondrial toxicity and subsequent apoptotic events, as detected by time-lapse fluorescence microscopy (Figure 3b). BCL-XL most significantly delayed the CTL-induced loss of the mitochondrial transmembrane potential $\Delta\psi_m$ (Figure 3c). Moreover, BCL-XL prevented apoptotic blebbing and permeabilization of the cell membrane at least for the assay duration of 5 h (Figure 3c). Expression of DN-FADD resulted in a less pronounced delay in apoptotic membrane changes, which is consistent with an additional contribution of death receptor signalling to apoptosis induced by p53-specific CTL (Figures 2 and 3c). As expected, BCL-XL expression conferred a strong protection of our MEF cancer cells against apoptosis induced by radiation or cytotoxic drugs (Supplemental Figure 1b). Of the four apoptosis inhibitors used in our studies, only BCL-XL enabled transformation of murine fibroblasts by a single oncogene (Supplemental Figure 1c). Taken together, prevention of MOMP and caspase activation by BCL-XL seems to provide a strong selective advantage for cancer cells in terms of oncogenic transformation, radiation or drug resistance, as well as resistance against CTL-mediated cytotoxicity *in vitro*.

BCL-XL abolishes CTL-mediated tumour suppression *in vivo*

To study whether antiapoptotic BCL-XL also confers *in vivo* resistance against CTL-mediated tumour suppression, murine fibrosarcoma tumours were established by subcutaneous injection of MEF in NOD/SCID mice. MEF expressing BCL-XL exhibited no growth advantage over vector-expressing control MEF *in vitro* (Figure 4a), and BCL-XL or vector MEF fibrosarcomas developed at similar rates *in vivo*, resulting in palpable flank tumours within 2 weeks of injection (Figure 4b). Comparing the *in vivo* growth of established tumours expressing BCL-XL or control vector in NOD/SCID mice (Figure 5a), we found that a single adoptive transfer of allo-A2K^b-reactive CTL strongly reduced the growth of vector-expressing fibrosarcomas. In contrast, the expression of BCL-XL impaired this tumour-suppressive activity of allo-A2K^b-reactive CTL *in vivo* (Figure 5b). To confirm and extend this observation, we compared the tumour-suppressive activity of CTL reactive to the HLA-A*0201-presented human p53 (149–157) epitope (A2 p53.149) on vector and BCL-XL-expressing fibrosarcomas. To control for nonspecific T-cell effects, H-2D^b/influenza PR8 nucleoprotein (366–374)-specific CTL (D^{b}NP) were used. Again, the adoptive transfer of TAA-specific CTL resulted in a substantial growth retardation of vector tumours, whereas BCL-XL-expressing fibrosarcomas exhibited resistance against tumour-reactive CTL *in vivo* (Figure 5c). The expression of transgenic BCL-XL or its absence was confirmed by immunoblotting analysis of fibrosarcomas obtained at termination of the experiment (Figure 5d). Hence, antiapoptotic BCL-XL proved to confer resistance of established murine fibrosarcomas against the growth suppression by adoptively transplanted, tumour-reactive CTL *in vivo*.

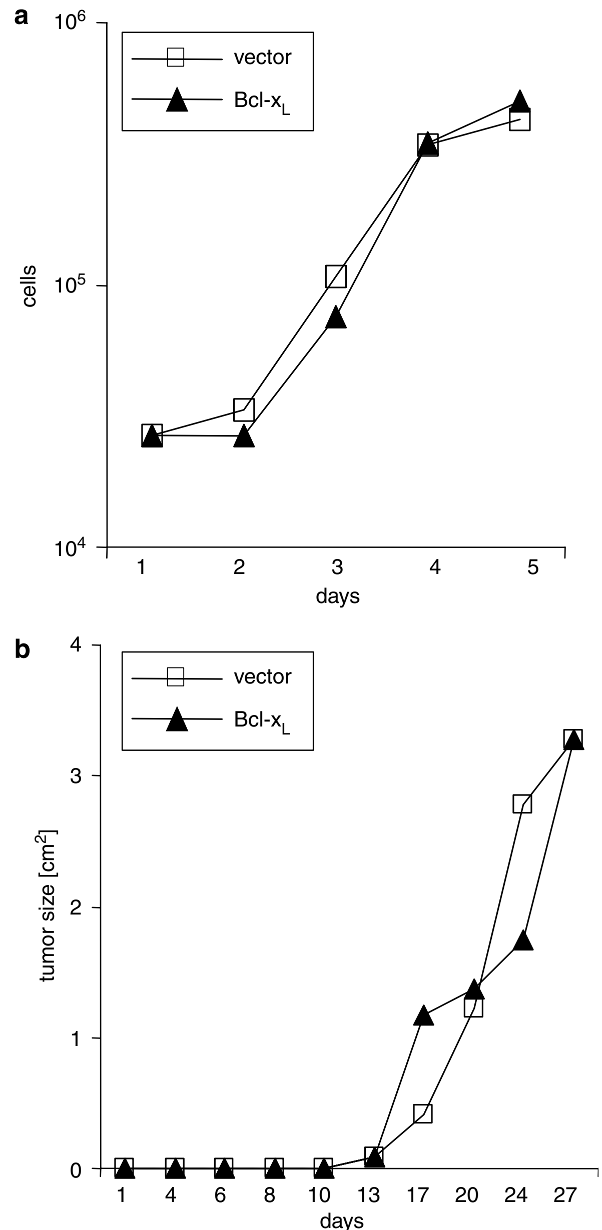

Figure 4 BCL-XL confers no growth advantage to p53$^{-/-}$ A2K^b MEF *in vitro* and *in vivo*. (**a**) *In vitro* growth curves of *E1A*/H-*ras*-transformed p53$^{-/-}$ A2K^b MEF expressing Bcl-XL (closed triangles) or control vector (open boxes). (**b**) *In vivo* growth of *E1A*/H-*ras*-transformed p53$^{-/-}$ A2K^b MEF expressing BCL-XL (closed triangles) or control vector (open boxes) following subcutaneous injection of 5×10^6 cells in NOD/SCID mice. Bidimensional tumour sizes were determined using a caliper. Mean values of at least two independent experiments are shown

Discussion

During malignant transformation, cancer cells have to evade several tumour suppressor mechanisms, including apoptotic

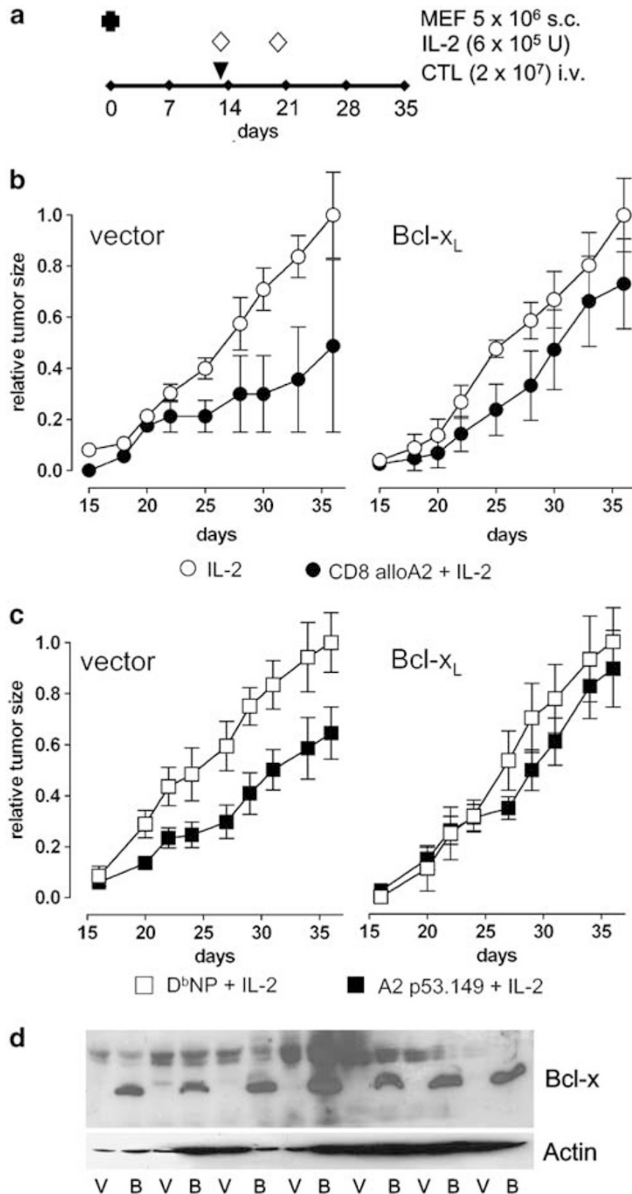

Figure 5 BCL-XL protects fibrosarcomas against the suppression of tumour growth by adoptively transplanted CTL *in vivo*. (**a**) Schematic representation of the course of the experiments. (**b**) Growth of established A2K^b MEF fibrosarcomas expressing BCL-XL (right panel) or control vector (left panel) in NOD/SCID mice following the adoptive transfer of allo-A2K^b-reactive CTL plus IL-2 (closed circles), or IL-2 treatment alone (open circles). Bidimensional tumour sizes were normalized to the maximum size of control tumours treated with IL-2 alone, and mean values $\pm$ S.E. of 16 tumours in eight mice are given. (**c**) Growth of established MEF fibrosarcomas expressing BCL-XL (right panel) or control vector (left panel) in NOD/SCID mice following the adoptive transfer of p53-reactive CTL (A2 p53.149) and IL-2 (closed boxes), or irrelevant CTL (D^{b}NP) and IL-2 treatment (open boxes). Bidimensional tumour sizes were normalized to the maximum size of control tumours treated with D^{b}NP CTL plus IL-2, and mean values $\pm$ S.E. of 28 tumours in 14 mice are given. (**d**) Immunoblotting of tumour cell extracts obtained from 14 fibrosarcomas of seven mice from experiment (**c**) using primary antibodies against Bcl-X and Actin. 'V' denotes control vector, and 'B' denotes BCL-XL expressing tumours

cell death. In general, this is achieved by genetic and/or epigenetic inactivation of key molecules involved in these processes, such as the p53 tumour suppressor protein and its positive regulator ARF, or amplification of its negative regulator MDM2. More than a decade ago, it has been experimentally demonstrated that inactivation of the p53 pathway not only enables oncogenic transformation but also confers resistance to apoptosis induced by clinically applied cytotoxic therapies including γ-radiation and anticancer drugs.[9] More recently, the molecular pathways how p53 signals apoptosis have been characterized. In most cell types, this is achieved through the proapoptotic members of the BCL-2 protein family such as the 'BH3-only' proteins PUMA and NOXA, as well as the 'BH123' protein BAX.[32–35] The coordinated action of these BCL-2 family proteins regulates MOMP and the subsequent release of mitochondrial apoptogenic factors into the cytoplasm,[18] which in turn enable the formation of the APAF-1 apoptosome complex to activate caspase-9. Active caspase-9 then cleaves and activates the executioner caspase zymogens to kill the cell ultimately.[19] Results from experimental cancer models suggest that defects in the apoptotic signal transduction downstream of p53 might facilitate oncogenic transformation and confer drug resistance.[6,8] Accordingly, functional blocks at the level of the BCL-2 family proteins and at the apoptosome level have been described in cancer cell lines and primary tumour samples.[36–41]

As a consequence, rationally designed therapies should aim to activate cancer cell apoptosis via mechanisms that bypass these genetic blocks in the p53/BAX/APAF-1/caspase-9 pathway. One possible strategy is the activation of death receptors, such as CD95/Fas/APO-1 or the TRAIL receptors, by recombinant ligands or activating antibodies.[42] At least in some cell types, death receptor activation and subsequent DISC formation are sufficient to activate directly effector caspases and induce apoptosis. However, in 'type II cells', a mitochondrial amplification step, which can be blocked by the overexpression of BCL-2 or combined deficiencies of BAX and BAK, seems required for effective caspase activation via the death receptor pathway.[43] Moreover, death receptor activation is nonspecific and thus may result in a substantial toxicity of nonmalignant tissues.[44,45] In contrast, TAA-specific CTL only lyse tumour cells, which present the respective target antigen within the context of the proper class I major histocompatibility complex molecule. Further, CTL are thought to induce target cell apoptosis via several parallel pathways, including death receptor activation, perforin/granzyme B and granzyme A,[12–16] and thus should be able to overcome single apoptosis defects selected during malignant transformation.

Surprisingly, our present results indicate that the expression of some inhibitors of apoptotic caspase activation not only results in resistance to radiotherapy and cytotoxic drugs but can also protect cancer cells against CTL-based immunotherapy. Depending on the type of CTL and experimental antigen employed, inhibitors of the mitochondrial pathway of caspase activation, such as BCL-XL and DN-Casp-9, as well as inhibition of death receptor-mediated apoptosis by DN-FADD suppressed CTL-induced cancer cell lysis in short-term assays. However, only BCL-XL expression translated into a significant advantage in terms of proliferative survival *in vitro*. This discrepancy could be explained by the prevention of the release of several mitochondrial apoptogenic factors, such as cytochrome *c*, SMAC/DIABLO or

HtrA2/OMI, through BCL-XL. In contrast, DN-Casp-9 is thought to interfere selectively with APAF-1-dependent caspase activation, and seems insufficient to block granzyme B-induced apoptosis *in vitro*.[46,47] This is in agreement with recent genetic evidence demonstrating that the loss of postmitochondrial activators of apoptosis, such as caspase-9 or APAF-1, fails to accelerate Myc-induced tumorigenesis in a murine model of lymphoma development or to prevent proliferative cell death in MEF and haematopoietic cells.[48,49] Moreover, BCL-XL also prevents death receptor-mediated caspase activation in MEF cancer cells, which behave like type II cells requiring mitochondrial amplification of the caspase-8 signal (not shown). DN-FADD, however, exclusively blocks the death receptor pathway,[20] leaving the mitochondrial and granule-mediated pathways intact. Finally, BCL-XL like BCL-2 prevents nonapoptotic mitochondrial death pathways,[50] which could impact on the outcome of CTL-induced reduction of proliferative survival. In conclusion, MOMP regulated by the BCL-2 family proteins is a rate-limiting step in CTL-mediated cancer cell apoptosis in our experimental system. The expression of antiapoptotic BCL-2 family proteins adds to established immune escape mechanisms of tumour cells, such as the expression of $FLIP_L$ or serpins.[51–53]

In extension of our results obtained *in vitro*, MEF fibrosarcomas expressing BCL-XL also exhibited protection against tumour suppression by adoptively transferred, allo-reactive and TAA-specific CTL *in vivo*. This system, which is regarded a valid *in vivo* model for the study of the activity of TAA-specific CTL,[54,55] mimics the therapeutic principle of graft-versus-tumor (GvT) effect elicited during ASCT. Currently, ASCT is clinically explored in a variety of drug-resistant cancers, including refractory or high-risk lymphoma, myeloma, breast cancer and renal cell cancer. Such drug-resistant cancers frequently exhibit upregulation of antiapoptotic proteins,[11] and the survival advantage of resistant tumour cell clones under the selective pressure of conventional cytotoxic cancer therapies may very well result in cross-resistance against immune-mediated tumour cell destruction. Our results demonstrate that the efficacy of ASCT especially in advanced-stage cancer patients may be hampered by such 'crossresistance' mechanisms.

In conclusion, despite their sophisticated armament, CTL can be seriously impaired in their ability to destroy drug-resistant cancer cells. The expression of $FLIP_L$ or serpins can protect cancer cells against CTL-mediated apoptosis.[51–53] However, those molecules fail to provide resistance against cytotoxic cancer therapies, which signal caspase activation via the 'mitochondrial' pathway. Our present data demonstrate that further genetic inhibitors of caspase activation are sufficient to confer resistance to adoptively transplanted, tumour-reactive CTL. We identify MOMP, which is initiated by granzyme B and caspase-mediated cleavage of BID, as a rate-limiting 'bottle-neck' in CTL-induced apoptosis. At least in the present experimental system, parallel pathways triggered by death ligands or direct activation of caspases and nucleases via perforin and granzymes A and B were insufficient to compensate for the shut down of the 'mitochondrial' pathway of caspase activation and caspase-independent death mechanisms by BCL-XL. Interestingly, MOMP is a key step in radiation- and drug-induced apoptosis of cancer cells, and also contributes to tumour suppression via the p53 pathway. Hence, genetic alterations selected during oncogenesis and cancer treatment can confer 'crossresistance' to CTL-induced tumour destruction. Combining CTL-based therapies with agents directly targeting the BCL-2 family proteins[56,57] or postmitochondrial caspase activators[58,59] could be valid strategies to overcome such 'immunoresistance' of cancer.

Materials and Methods

Cell lines

Fibroblasts were generated from day E14 embryos of $p53^{-/-}$ $A2K^b$ transgenic mice[60] following standard techniques, and the respective genotypes were confirmed. These murine embryo fibroblasts (MEF) were transformed by parallel transduction with retroviral vectors expressing *E1A* and H-*ras* (gifts from Dr. S Lowe) as described previously.[35] A cDNA encoding the human $p53^{V143A}$ mutant was subcloned into the retroviral vector pBabeBleo to transduce sequentially the oncogene-transformed MEF. Several antiapoptotic cDNA (encoding BCL-XL, DN-FADD, DN-Casp-9 and $XIAP^{\Delta RING}$) were subcloned into the vector pMxIG (a gift from Dr. T Kitamura), and inserts were confirmed by sequencing. Retroviral virions were generated by transient cotransfection of 293T cells with the helper plasmid pCL_Eco.[61] HLA-A*0201/human p53 (149–157)- and (264–272)-specific CTL derived from A2 transgenic mice, HLA-A*0201/ influenza matrix (58–66)- and H-2D^b/nucleoprotein (366–374)-specific CTL derived from human CD8 $\times$ $A2K^b$ transgenic and C57BL/6 mice, respectively, as well as allo-$A2K^b$-reactive CTL from human CD8 transgenic mice, have been described previously.[22,23]

Immunoblotting

Immunoblotting was performed as described previously[35] using primary antibodies against caspase-9 (9CSP02, Chemicon), FADD (rabbit antiserum, Calbiochem), XIAP (rabbit antiserum, R&D Systems), BCL-X (2H12, Pharmingen), Actin (C4, ICN) and p53 (CM5, Novocastra).

Cytotoxicity and apoptosis assays

The 5 h ^{51}Cr release cytotoxicity assays were carried out as described.[22] Apoptosis was detected by cell cycle analysis following staining with propidium iodide (PI). For detection of caspase activation, MEF were loaded with the fluorescent marker DiI (Molecular Probes) and then cocultured for 30–45 min with CTL. Following incubation with FITC-VAD (Oncogene), the fraction of $DiI^+/FITC^+$ cells was determined by flow cytometry. To assay proliferative survival, 5000 adherent MEF were incubated in 96-well plates with CTL effectors at the indicated E : T ratios for 4.5 h. Following removal of CTL, MEF were harvested and replated in 35 mm dishes for a 7-day culture period. The resulting colonies were fixed, stained and counted.

Time-lapse microscopy

GFP-expressing MEF targets were plated on Thermanox chamber slides (Nunc) and stained with the mitochondrial marker tetramethylrhodamine ethylester (TMRE, 75 nM for 30 min, Molecular Probes). CTL (A2 p53.149) resuspended in phenol-free medium supplemented with PI (50 μg/ml) were added at an E : T of 10, and the medium was overlaid with mineral oil.

Time-lapse images (excitation frequencies 488 and 560 nm) were taken in 2 min intervals on an Olympus IX-70 inverted microscope with a heated stage and a digital imaging system for 300 min. Analyses were performed using the TILL vision 4.0 software.

Adoptive CTL transfer into tumour-bearing NOD/SCID mice

Irradiated (150 rad) NOD/SCID mice received bilateral subcutaneous injections of 5×10^6 MEF (BCL-XL MEF right flank, vector MEF left flank). Following the outgrowth of palpable fibrosarcomas, the mice were treated with single tail vein injections of 2×10^7 CTL resuspended in saline (day 13) as well as two subcutaneous doses of 6×10^5 IU recombinant human interleukin-2 (IL-2) resuspended in saline and incomplete Freund's adjuvant (days 13 and 20). Tumour size was measured bidimensionally using a calliper.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)