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# MAPK/ERK signaling in activated T cells inhibits CD95/Fas-mediated apoptosis downstream of DISC assembly

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**When T cells are activated, the expression of the CD95 ligand is elevated, with the purpose of inducing apoptosis in target cells and to later eliminate the activated T cells. We have shown previously that mitogen-activated protein kinase (MAPK or ERK) signaling suppresses CD95-mediated apoptosis in different cellular systems. In this study we examined whether MAPK signaling controls the persistence and CD95-mediated termination of an immune response in activated T cells. Our results show that activation of Jurkat T cells through the T cell receptor immediately suppresses CD95-mediated apoptosis, and that this suppression is mediated by MAPK activation. During the phase of elevated MAPK activity, the activation of caspase-8 and Bid is inhibited, whereas the assembly of a functional death-inducing signaling complex (DISC) is not affected. These results explain the resistance to CD95 responses observed during the early phase of T cell activation and suggest that MAPK-activation deflects DISC signaling from activating caspase-8 and Bid. The physiological relevance of the results was confirmed in activated primary peripheral T cells, in which inhibition of MAPK signaling markedly sensitized the cells to CD95-mediated apoptosis.**
*Keywords*: apoptosis/death receptor/MAP kinase/T cell activation/T cell receptor

## Introduction

Apoptosis is essential in the control of immune responses, embryonic development and tissue homeostasis in the adult. In particular, CD95 (Fas/APO-1)-mediated apoptosis plays an important role in the immune system, both in the negative selection of T cells and in eliminating target cells as well as unwanted lymphocytes during the immune response (Nagata and Golstein, 1995). CD95 is a 48 kDa large glycoprotein (Itoh *et al*., 1991; Oehm *et al*., 1992) belonging to the tumor necrosis factor receptor (TNF-R) superfamily. Oligomerization of CD95 by the CD95 ligand (CD95L/FasL; Suda *et al*., 1993) activates the apoptotic pathway (Itoh *et al*., 1991; Oehm *et al*., 1992) by recruiting a set of proteins that form the death-inducing signaling complex (DISC; Kischkel *et al*., 1995). The DISC contains several proteins with distinct functions, among them a cytosolic adaptor protein, Fas-associated protein with death domain (FADD or MORT1; Chinnaiyan *et al*., 1995; Boldin *et al*., 1996). The death domain (DD) of CD95 binds to the C-terminal DD of FADD, whose N-terminal death effector domain (DED) in turn recruits caspase-8 (Chinnaiyan *et al*., 1995; Muzio *et al*., 1996), which is thereby autoproteolyzed and activated (Medema *et al*., 1997). Recently, a molecular link between DISC-activated caspase-8 and the mitochondria was described (Li *et al*., 1998; Luo *et al*., 1998). In these studies, the Bcl-2 family protein Bid was shown to be a direct target for caspase-8. Cleavage of the p22 Bid by caspase-8 results in the formation of a truncated p15 Bid protein, which directly affects mitochondria and releases cytochrome *c*. Several effector caspases are then cleaved and activated, thereby amplifying the caspase cascade (Cryns and Yuan, 1998). This mitochondrial activation pathway has been shown to be essential for CD95-mediated apoptosis in certain cell types, termed type II cells (Scaffidi *et al*., 1999b).

Resting peripheral T cells are activated during an immune response by repeated stimulation of both the T cell receptor (TCR) and the co-receptors, with specific antigens, mitogens or antibodies. This activation results in increased expression of several genes, including CD95 and CD95L (Klas *et al*., 1993; Brunner *et al*., 1995). A major role for the latter is activation of apoptosis in unwanted target cells, such as tumor cells (Nagata and Golstein, 1995). When the activated T cells have accomplished their task of killing self-altered or virus-infected cells, they themselves undergo CD95L-mediated activation-induced cell death (AICD). AICD is induced within 1 day of OKT3 stimulation in Jurkat T cells and in several murine T cell hybridoma cell lines, and within 5–7 days in peripheral T cells (Brunner *et al*., 1995; Dhein *et al*., 1995; Ju *et al*., 1995). AICD is a major mechanism for removing activated T cells after they have accomplished the task of clearing virus-infected or tumorigenic cells from the host. Therefore, the precise regulation of CD95-mediated apoptosis is of crucial importance to ensure elimination of target cells as well as termination of the immune response. Since AICD occurs long after the CD95L levels start to increase, there has to be specific mechanisms responsible for protecting the cells against CD95 stimulation. Upregulation of the apoptosis-inhibitory protein c-FLIP (Irmler *et al*., 1997; Tschopp *et al*., 1999) and


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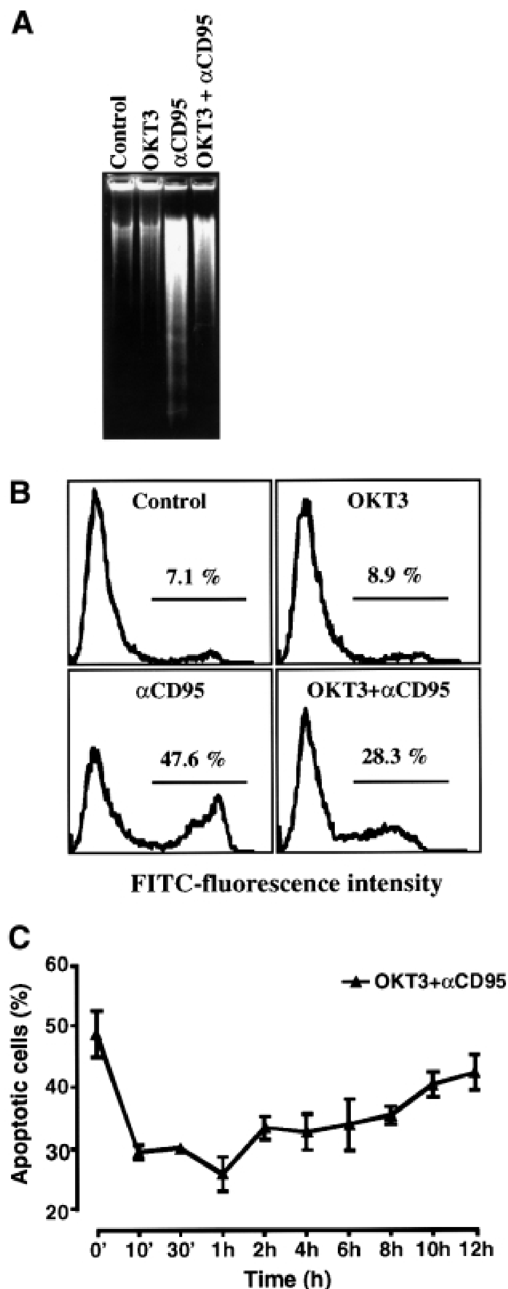



**Fig. 1.** Activation of Jurkat T cells with an agonistic CD3 antibody, OKT3, suppresses CD95-mediated apoptosis. Jurkat T cells were incubated with medium alone, immobilized anti-CD3 (OKT3) antibody (100 µg/ml), anti-CD95 (anti-Fas, 100 ng/ml) or pre-incubated for 30 min with immobilized OKT3 before addition of anti-CD95. After 2 h aliquots of cells were analyzed by either (**A**) one-stage DNA gel electrophoresis or (**B**) annexin V–FITC staining, and the proportion of apoptotic nuclei was determined using a FACScan flow cytometer. Bars indicate the percentage of cells with exposed phoshatidylserine (FITC positive), a feature that is characteristic of apoptotic cells. (**C**) The OKT3-mediated suppression of CD95-induced apoptosis lasts for 10–12 h after stimulation. Jurkat T cells were pre-incubated with immobilized OKT3 for the indicated time periods prior to incubation with anti-CD95 for 2 h and analyzed as in (A). The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

anti-apoptotic Bcl-family members (Peter *et al*., 1997) has been indicated to protect activated T cells from CD95-mediated apoptosis, but their expression does not always correlate with CD95 sensitivity (Scaffidi *et al*., 1999a). Therefore, it is difficult to develop a paradigm based solely on the expression of these proteins, which would explain the protection observed during the early phase of T cell activation. While there is evidence for a role of phosphorylation-mediated signaling in regulation of CD95 responses (Holmström *et al*., 1998, 1999), little is known about the role of phosphorylation in the suppression of CD95-mediated apoptosis during T cell activation.

A possible signaling candidate that could protect activated T cells from AICD is the mitogen-activated protein kinase (MAPK or ERK) pathway, a major regulator of cell activation, proliferation and differentiation (Lewis *et al*., 1998). Ligation of the TCR activates two major signaling cascades, namely the MAPK and calcineurin pathways, both of which are required for T-cell-specific transcriptional activation. The paradigm of MAPK as an inhibitory pathway in regulation of CD95-mediated apoptosis is supported by studies showing suppression of apoptosis by MAPK activation (Xia *et al*., 1995) and by our studies showing that MAPK specifically protects cells from CD95-induced apoptosis (Holmström *et al*., 1998, 1999). Furthermore, signaling through the TCR has previously been shown to antagonize CD95-mediated apoptosis (Klas *et al*., 1993; Peter *et al*., 1997). It is also important to know where in the CD95-signaling pathway TCR signaling is exerting its effects. This is needed in order to determine whether the cells still maintain their normal functions, or whether they shift to some other functional state when CD95 is stimulated but no apoptotic response is triggered. We wanted, therefore, to investigate more closely the role of the MAPK pathway in protecting recently activated T cells from CD95-mediated apoptosis. In the present study we show that TCR-mediated MAPK activation suppresses CD95-mediated apoptosis during the early phase of T cell activation. This inhibition is protein synthesis independent and occurs after assembly of a functional DISC but before overall caspase-8 activation and cleavage of Bid. These results imply that direct MAPK-mediated signaling, in addition to the known inhibitory proteins, also participates in regulating the functions and responses of CD95.

## Results

### *Activation of Jurkat T cells with the agonistic CD3 antibody, OKT3, suppresses CD95-mediated apoptosis*

To determine how activation of the CD3–TCR complex affects CD95-mediated apoptosis, we pre-treated Jurkat T cells with an immobilized agonistic CD3 antibody, OKT3, and analyzed whether OKT3 suppressed two characteristic signs of apoptosis: the formation of oligonucleosomal DNA fragments (Figure 1A) and phosphatidylserine exposure on the cell membrane (Figure 1B). Pre-treatment of Jurkat T cells with OKT3 clearly suppressed the amount of apoptotic cells by ~50%. Furthermore, the duration of OKT3-induced suppression of CD95-mediated apoptosis was analyzed and shown to last for 10 h (Figure 1C).

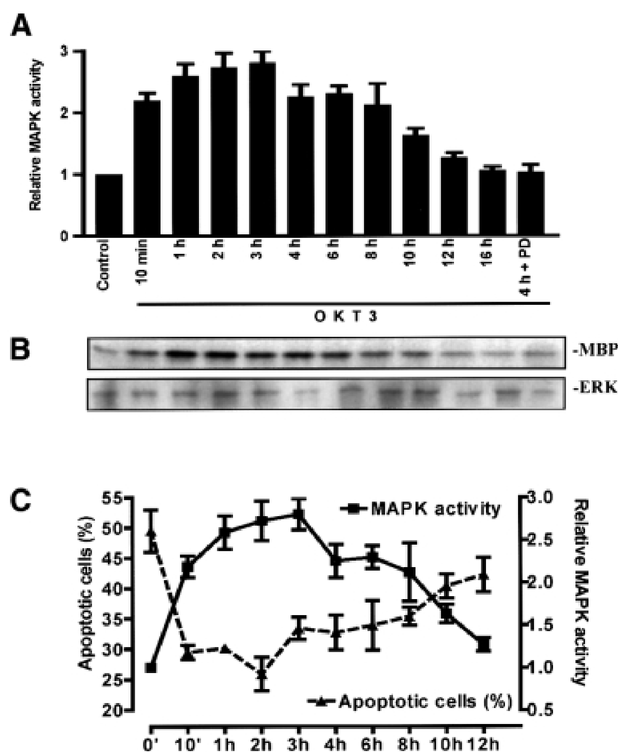



**Fig. 2.** The kinetics of MAPK activation in Jurkat T cells after OKT3 stimulation corresponds to the observed suppression of CD95-mediated apoptosis. MAPK activation was followed in cell extracts incubated with medium alone, in the presence of immobilized OKT3 or in the presence of the MKK1 inhibitor PD 98059 (30 µM) + OKT3, and the activation was determined by an immunocomplex kinase assay. (**A** and **B**) A representative autoradiograph of the immunocomplex kinase assay together with an immunoblot of the immunoprecipitated ERK2, to verify presence of the MAPK, is shown. Multiple samples at the different time points were quantified by phosphoimager analysis. (**C**) A close correlation between MAPK activity and the amount of apoptotic cells was obtained when the data from the MAPK assays and the percentage of apoptotic cells, treated as described above, were plotted against the incubation time with OKT3. (B and C) The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

### *OKT3-mediated MAPK activation corresponds to the observed suppression of CD95-mediated apoptosis*

Since the TCR is a potential target for MAPK, we compared the kinetics of MAPK activation with the observed suppression of CD95-mediated apoptosis by OKT3. A MAPK assay showed that stimulation of Jurkat T cells with OKT3 induces a rapid increase in MAPK activity starting 10 min after stimulation of CD3 and lasting up to 10–12 h (Figure 2A). The MAPK activity peaked at 1–4 h, being ~2.5-fold higher than the control values (Figure 2B). This activation was not due to increased synthesis of MAPK as the MAPK protein levels remained constant following CD95 stimulation (Figure 2B). The OKT3-induced MAPK activation was completely inhibited by pre-treatment of Jurkat T cells with the specific MKK1 inhibitor, PD 98059 (Alessi *et al.*, 1995), before triggering CD95 (Figure 2A). Furthermore, there was a close correlation between OKT3-mediated MAPK activation and suppression of CD95-mediated apoptosis (Figure 2C).

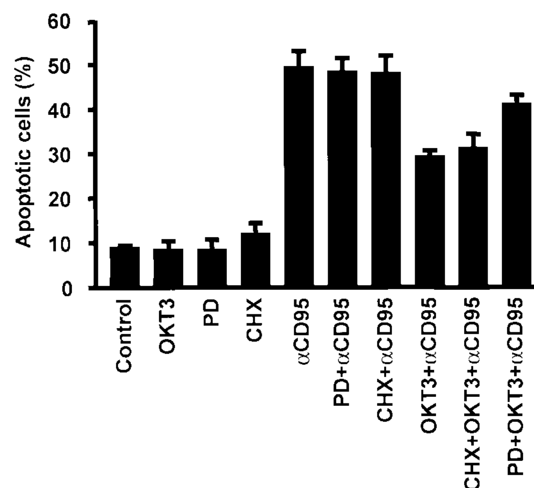



**Fig. 3.** OKT3-mediated suppression of CD95-induced apoptosis in Jurkat T cells is independent of protein synthesis and mediated through MAPK activation. Annexin V–FITC staining was used as an indicator for the amount of apoptotic cells. Cells were cultured for 2 h with medium alone, in the presence of anti-CD95 (anti-Fas), immobilized OKT3, PD 98059, CHX (100 µg/ml), OKT3 + anti-CD95, CHX + anti-CD95, CHX + OKT3 + anti-CD95 or PD 98059 + OKT3 + anti-CD95. The cells were pre-incubated with immobilized OKT3 as previously described and/or for 10 min with CHX prior to incubation with anti-CD95. The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

### *The OKT3-mediated suppression of CD95-mediated apoptosis is MAPK dependent and independent of protein synthesis*

As we have shown previously that MAPK signaling suppresses CD95-mediated apoptosis (Holmström *et al.*, 1998, 1999), we wanted to determine whether MAPK activation is responsible for the observed resistance of recently activated Jurkat T cells to CD95-mediated apoptosis, and if this suppression requires protein synthesis. The MAPK dependence was demonstrated by pre-treatment of the cells with PD 98059, which abolished the protective effect of OKT3 on CD95-mediated apoptosis (Figure 3). However, cycloheximide (CHX) treatment had no effect, as cells pre-treated with immobilized OKT3 were equally insensitive to CD95-mediated apoptosis both in the presence and absence of CHX (Figure 3), indicating that the process is independent of protein synthesis. The MAPK-dependent protection was further corroborated by transient transfection of Jurkat T cells with a dominant-negative MKK1 containing a hemagglutinin (HA) tag (Mansour *et al.*, 1996). The transfected cells were then visualized by immunofluorescence staining of HA and the nuclear morphology by DNA staining (Figure 4A). The protective effect of OKT3 on CD95-mediated apoptosis was abolished in the cells expressing dominant-negative MKK1 (Figure 4B). Taken together, these results show that the observed suppression of CD95 is MAPK dependent and protein synthesis independent.

### *Inhibition of MAPK signaling sensitizes activated primary T cells to CD95-mediated apoptosis*

Resting T cells express low levels of CD95 on the cell surface. One day after activation of peripheral T cells the levels of CD95 are upregulated, and on day 5–7, when

grown continuously in the presence of interleukin-2, the cells show a CD95-sensitive phenotype (Klas *et al*., 1993; Peter *et al*., 1997). It is therefore interesting to examine whether MAPK signaling is able to modulate the sensitivity of T cells to CD95-mediated apoptosis. For this purpose we activated peripheral blood T cells with 1 μg/ml phytohemagglutinin (PHA) for 5–6 days prior to triggering CD95 in the absence or presence of PD 98059 (Figure 5). Quite interestingly, the specific MKK1 inhibitor, PD 98059, clearly sensitized T cells to CD95-mediated apoptosis. Our results imply that primary T cells are relatively insensitive to CD95-mediated apoptosis for 5 days after activation. Suppression of MAPK activation, however, sensitizes them to CD95-mediated apoptosis (Figure 5).

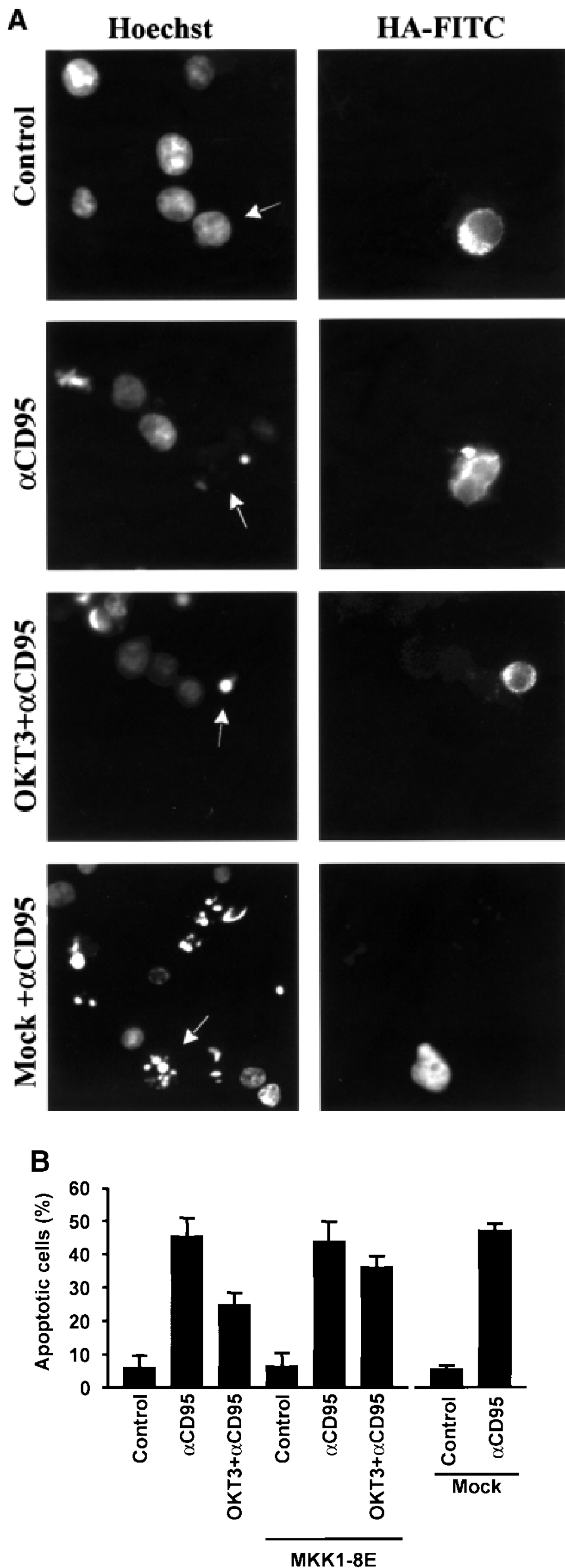



**Fig. 4.** Dominant-negative MKK1 abolishes the anti-CD3-mediated protective effect on CD95-mediated apoptosis. (**A**) Representative immunofluorescence micrographs of cells transfected with a dominant-negative (MKK1-8E) construct and incubated for 2 h with anti-CD95 in the absence or presence of OKT3. Hoechst staining was used to detect alterations in the nuclei and a monoclonal HA antibody linked to an FITC-conjugated secondary antibody was used to detect the presence of the HA-tagged MKK1 in transfected cells. The arrows indicate the transfected cells. Mock transfected cells were treated as indicated above with or without stimulation of CD95. (**B**) Percentage of apoptosis in transfected cells after treatment with anti-CD95. The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

### *Activation of Jurkat T cells by OKT3 does not affect the levels of apoptotic inhibitory proteins, Bcl-2, Bcl-$x_L$ and FLIP*

Although our results indicate that the OKT3-mediated suppression of apoptosis is protein synthesis-independent, we wanted to examine more closely the effect of OKT3 treatment on some of the known anti-apoptotic proteins. In order to examine whether Bcl-2, Bcl-$x_L$ and FLIP protein levels were increasing, we performed immunoblotting of the respective proteins after treatment of Jurkat T cells with OKT3 (Figure 6A). Immunoblotting of Bcl-2, Bcl-$x_L$ and FLIP showed that all these proteins are constitutively expressed in Jurkat T cells, and that there was no significant increase in the amount of these proteins. This indicates that these proteins are not affected by OKT3 treatment and it is unlikely that they are involved in the observed suppression of CD95-mediated apoptosis in Jurkat T cells.

### *Activation of MAPK by TPA does not alter the distribution of the CD95*

One possible mechanism by which MAPK activation could affect the CD95 response is by altering the levels of the CD95 protein (Peli *et al*., 1999) or by changing the cellular compartmentalization of the receptor. It has indeed been indicated that MAPK signaling leads to phosphorylation and internalization of the TNF-R1 (Cottin *et al*., 1999). Our previous studies, however, did not indicate any alterations in the level or distribution of CD95 after activation of the MAPK signaling pathway (Holmström *et al*., 1999). To exclude the possibility that MAPK activation would affect the levels of receptor protein, we analyzed the presence of CD95 on the cell surface by FACS analysis in tetradecanoylphorbol 13-acetate (TPA)-treated samples, as TPA is a very efficient MAPK activator and also an efficient inhibitor of CD95-mediated apoptosis (Holmström *et al*., 1998). According

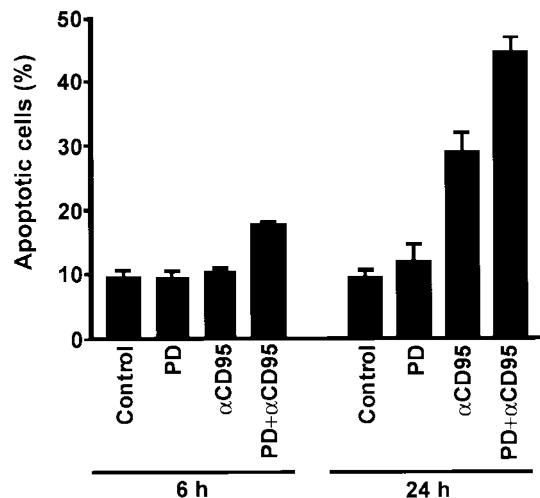



**Fig. 5.** Suppression of MAPK activation sensitizes activated human peripheral T cells to CD95-mediated apoptosis. Annexin V–FITC staining was used as an indicator for the amount of apoptotic cells. Activated T cells (day 5–6; $1 \times 10^6$ cells/ml) were cultured for 6 or 24 h with medium alone, in the presence of anti-CD95 (anti-Fas, 500 ng/ml), immobilized OKT3, PD 98059, OKT3 + anti-CD95 or PD 98059 + OKT3 + anti-CD95. The cells were pre-incubated with immobilized OKT3 as previously described prior to incubation with anti-CD95. The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

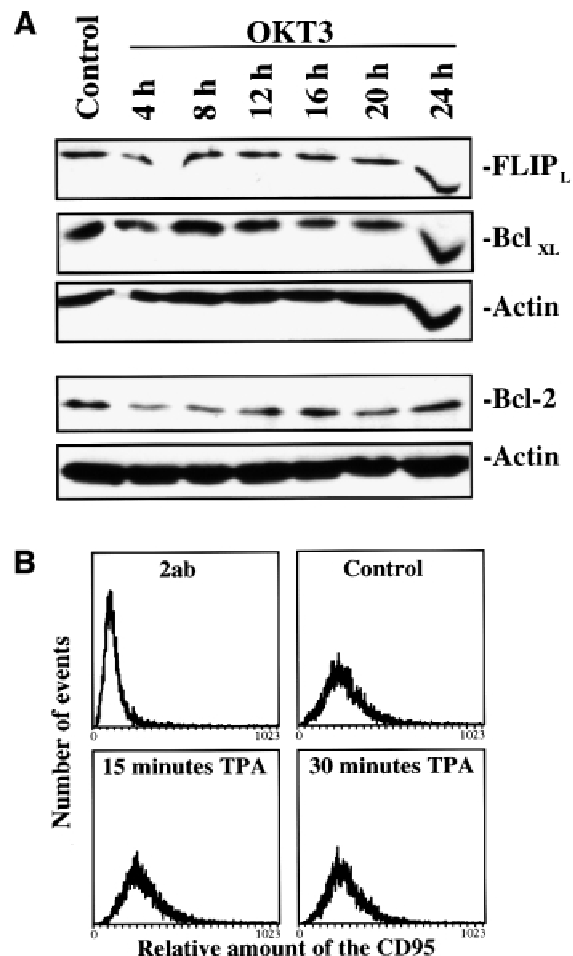
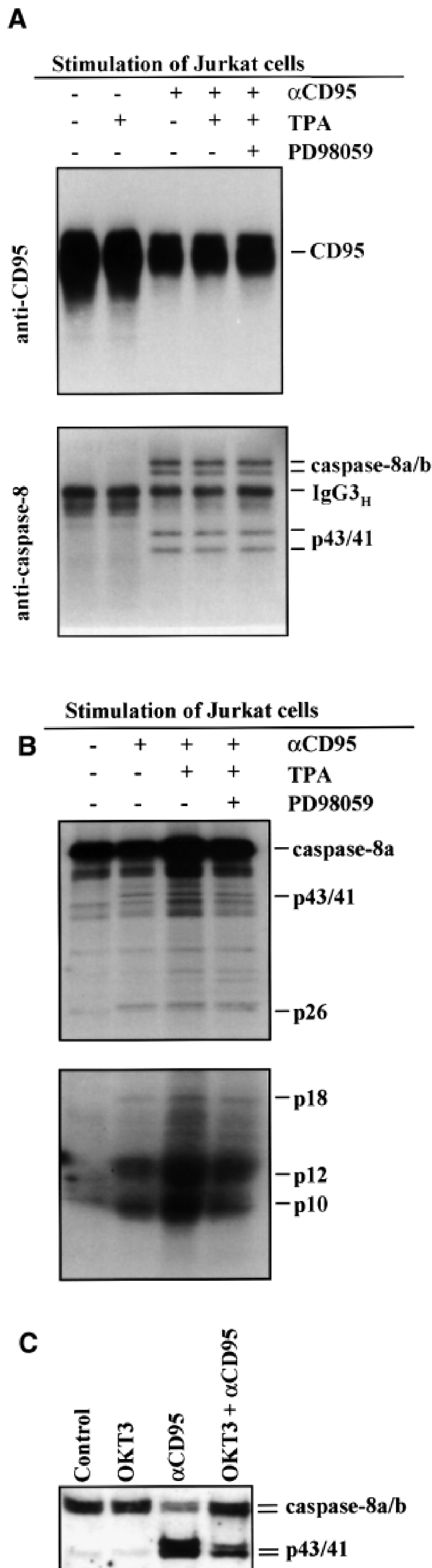



**Fig. 6.** OKT3 stimulation does not affect Bcl-$x_L$, Bcl-2, c-FLIP$_L$ and CD95 levels. (**A**) Jurkat T cells were treated with immobilized OKT3 for the indicated time points and the amount of proteins known to affect apoptosis was assessed by immunoblotting with specific antibodies to Bcl-$x_L$, Bcl-2 and c-FLIP$_L$. Equal loading was confirmed by immunoblotting of the same gel with an actin-specific antibody. A representative immnunoblot from three experiments is shown. (**B**) To measure the amount of CD95 on the cell surface after MAPK activation, Jurkat T cells were treated as indicated in the figure. The amount of CD95 was then assessed by staining with a specific CD95 antibody (anti-APO-1) followed by an FITC-conjugated secondary antibody and analyzed by flow cytometry. As a negative control we used cells stained with the secondary antibody only.

to our results, CD95 levels on the cell surface were not altered by TPA treatment (Figure 6B). These results exclude alterations of CD95 levels or cellular distribution of CD95 as a mechanism behind MAPK-mediated suppression of CD95-induced apoptosis.

### *MAPK activation does not affect formation of a functional DISC but suppresses overall activation of caspase-8*

Previous studies have indicated that only a small amount of caspase-8 cleavage and activation is required to trigger the CD95-mediated apoptotic pathway in Jurkat T cells (Scaffidi *et al.*, 1998, 1999b). In order to examine at which level in the CD95-signaling pathway MAPK activity is suppressing apoptosis, we immunoprecipitated CD95 followed by immunoblotting for caspase-8 from the immunoprecipitates. Jurkat T cells are type II cells that require the mitochondrial amplification loop for execution of CD95-mediated apoptosis, presumably because they form only low amounts of the DISC (Scaffidi *et al.*, 1998, 1999b). The caspase-8 immunoblot from CD95 immunoprecipitates, using both unstimulated control cells and CD95-stimulated cells, shows that caspase-8 is recruited to the DISC (Figure 7A). However, there were no significant differences in recruitment of caspase-8 to the DISC even when MAPK was activated by TPA (Figure 7A). Hence, the DISC seemed to assemble regardless of whether the cells are undergoing apoptosis or not. To test whether the DISC in TPA-treated Jurkat T cells had the capacity to convert procaspase-8 into active caspase-8 subunits, we performed an *in vitro* caspase-8 cleavage assay (Medema *et al.*, 1997) with the DISC isolated from Jurkat T cells (Figure 7B). In this assay the DISC of unlabeled cells is immunoprecipitated, and $^{35}$S-labeled caspase-8/a is added. After incubation for 24 h at 4°C, caspase-8/a will be processed into active fragments if the DISC is active. By western blotting, procaspase-8, as part of the DISC, was barely detectable in the Jurkat T cells (Figure 7A). However, the caspase-8 enzymatic activity present in the immunoprecipitated DISC was sufficient to process and activate caspase-8 at the receptor level (Figure 7B), demonstrating that also the DISC of Jurkat T cells pre-treated with TPA prior to triggering of CD95 was functionally active. However, while the DISC is functional even following MAPK activation, the elevated MAPK suppressed overall caspase-8 activation, as measured by analysis of caspase-8 cleavage in the whole-cell lysates (Figure 7C). The immunoblot of caspase-8 showed a clear decrease in the amount of cleaved caspase-8 in cells pre-treated with OKT3 prior to triggering of CD95 when compared with CD95-stimulation alone, indicating that MAPK activation suppresses a general cleavage and




activation of caspase-8 (Figure 7C). This observation is in accordance with our previous studies, indicating that elevated MAPK activity suppresses caspase activation (Holmström *et al.*, 1998, 1999). In conclusion, we could not detect any difference in DISC assembly nor activity after MAPK activation, whereas activation of the cytoplasmic pool of caspase-8 was clearly inhibited.

### MAPK activation suppresses cleavage of Bid

To narrow down the entry point of the MAPK-mediated inhibitory signal into the CD95 apoptotic signaling pathway, we tested whether the cleavage of Bid would be affected by MAPK activation. Cleavage of the p22 Bid by caspase-8 results in the formation of a p15 Bid protein, which directly affects mitochondria and releases cytochrome *c* (Li *et al.*, 1998; Luo *et al.*, 1998). To this end, we treated the cells with TPA prior to CD95 triggering and tested whether the effect of TPA could be abolished by pre-treatment with the MKK1 inhibitor PD 98059 (Figure 8A). Activation of MAPK clearly reduced the formation of the p15 Bid formed at 2 h and this protective effect was abolished when the samples were pre-treated with PD 98059, suggesting that MAPK suppresses activation of Bid itself or a target that affects the processing of Bid. A further indication that the Bid-mediated mitochondrial amplification loop is inhibited came from experiments showing that the mitochondrial membrane potential stays unaffected in CD95-stimulated cells in the presence of TPA (data not shown).

### MAPK activation phosphorylates Bad on Ser112

In addition to suppressing activation and cleavage of Bid, MAPK could also have an effect on other proteins in the mitochondrial apoptosis signaling pathway. An interesting cytosolic substrate for the MAPK pathway is the pro-apoptotic protein, Bad. Bad is known to heterodimerize with Bcl-$x_L$ and Bcl-2, both of which suppress CD95-mediated apoptosis downstream of caspase-8 activation in Jurkat T cells (Li *et al.*, 1998; Luo *et al.*, 1998; Scaffidi *et al.*, 1998, 1999b). Bad has recently been shown to be phosphorylated by the MAPK pathway on Ser112, thereby inactivating Bad. This inactivated Bad is held by the 14-3-3 protein, freeing Bcl-$x_L$ and Bcl-2 to promote survival (Bonni *et al.*, 1999; Scheid *et al.*, 1999). We therefore wanted to study whether MAPK activation would result in phosphorylation of Bad on Ser112 in

**Fig. 7.** MAPK activation suppresses overall cleavage of caspase-8 but does not affect formation of a functional DISC. (**A**) Jurkat T cells were treated as indicated in the figure prior to immunoprecipitation of CD95 from the cell extracts. Subsequently the levels of immunoprecipitated CD95 and associated caspase-8 were detected by western blotting with appropriate antibodies after the respective treatments. A representative immunoblot from three experiments is shown. (**B**) Jurkat T cells were treated as indicated in the figure prior to immunoprecipitation of CD95 from the cell extracts. Immunoprecipitates were washed four times and incubated with *in vitro* translated $^{35}$S-labeled caspase-8/a. After 24 h the samples were analyzed on 15% SDS–PAGE. The upper part of the gel was exposed for 24 h and the lower part was exposed for 5 days. (**C**) Jurkat cells were treated as indicated in the figure and the degree of caspase-8 processing in the cells was monitored by western blotting with a caspase-8-specific antibody. Caspase activation can be observed as the appearance of the p43/41 active intermediate fragments of the caspase proforms.

Jurkat T cells. The level of Bad phosphorylation on the Ser112 residue was assessed by immunoblotting using a Ser112 phospho-specific Bad antibody. The lowest band in the Ser112 phospho-specific Bad immunoblot (Figure 8B, upper panel) corresponds to the specific band on the Bad immunoblot (Figure 8B, lower panel). Our results clearly show that TPA induces an increase in the phosphorylation of Bad on Ser112, which is abolished when the cells are pre-treated with the specific MKK1 inhibitor PD 98059 (Figure 8B). This indicates that the TPA-generated phosphorylation of Bad on Ser112 is MAPK dependent.

## Discussion

The sensitivity of T cells towards TCR-induced AICD varies during the course of an immune response. In the early phase, peripheral T cells are resistant to CD95-mediated apoptosis. However, after successful completion of this process, most of the activated T cells are removed by AICD. This implies that the resistance of T cells to CD95-mediated apoptosis is tightly regulated to ensure homeostasis in the immune system. In our model system, the activated Jurkat T cells showed MAPK-dependent resistance to CD95-mediated apoptosis immediately after T cell activation, independently of the expression levels of the previously implicated CD95 inhibitors FLIP and Bcl-$x_L$. In this regard, post-translational regulation of receptor functions is likely to be significantly faster and more dynamic than regulation through protein synthesis dependent mechanisms. This resistance to CD95-mediated apoptosis is important, as the recently activated T cell will encounter other T cells already expressing the CD95L. If the recently activated T cells would not be protected, they would undergo apoptosis before they had accomplished their tasks. In fact, CD95 stimulation during the early phases of T cell activation may be beneficial, as indicated by studies showing that stimulation of CD95 together with suboptimal stimulation of the TCR induces mitogenic stimulation (Alderson *et al*., 1993) and that maximal proliferation of T cells through the TCR requires co-stimulatory signals through CD95 (Suzuki and Fink, 1998). In further support for a role of CD95 in T cell growth are studies showing that T cells lacking FADD or expressing a dominant-negative mutant FADD show impaired TCR-stimulated proliferation and AICD (Newton *et al*., 1998; Walsh *et al*., 1998; Zhang *et al*., 1998).

### *MAPK activation suppresses CD95-mediated apoptosis during T cell activation*

When the time-frame of MAPK activation was related to the suppression of CD95-mediated apoptosis in Jurkat T cells, it was obvious that OKT3 induced a rapid MAPK activation and suppression of CD95-mediated apoptosis, both of which lasted as long as the MAPK activity stayed elevated. Interestingly, OKT3 stimulation for 24 h induced CD95L-mediated autocrine suicide in Jurkat T cells (Dhein *et al*., 1995). These observations are consistent with the hypothesis that T cell activation has a dual role in both inhibiting and promoting apoptosis. Our results indicate that MAPK activation mediates a protective signal during the initial phases of activation, which is turned off towards the end of the immune response to allow the apoptotic CD95 signal to be executed. The physiological significance of MAPK-dependent protection against CD95-mediated apoptosis was further corroborated by our results demonstrating that the specific MKK1 inhibitor, PD 98059, sensitizes peripheral blood T cells to CD95-mediated apoptosis. These data imply that MAPK activation plays an important role in suppressing CD95-mediated apoptosis during T cell activation, and in regulating the duration and strength of the immune response. This may also explain the above-mentioned implications of synergistic effects between CD95 and the TCR.

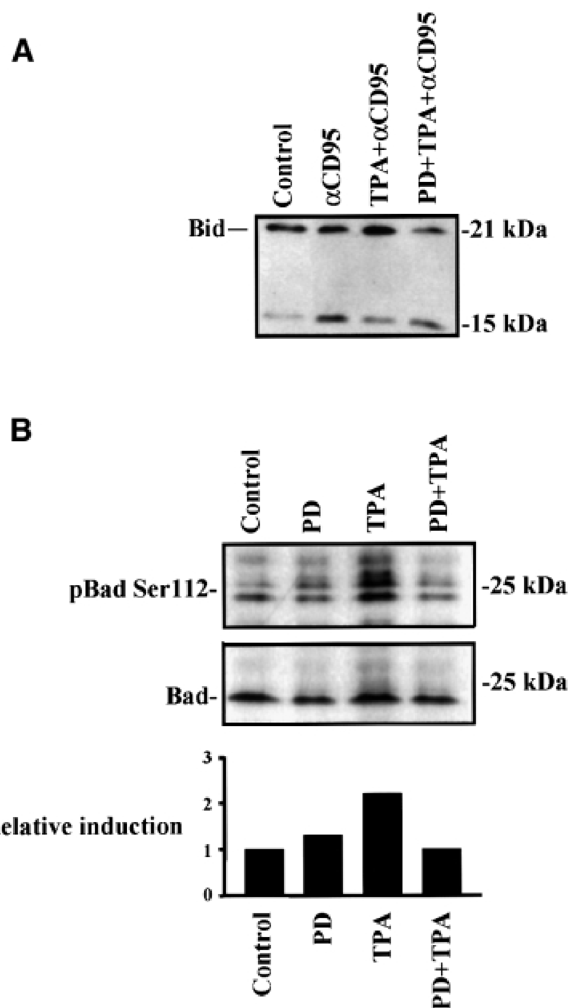



**Fig. 8.** MAPK activation suppresses cleavage of Bid and phosphorylates Bad on Ser112. (**A**) MAPK-mediated suppression of Bid cleavage. Jurkat T cells were treated as indicated in the figure for 2 h prior to immunoblotting for Bid. The amount of full-length Bid compared with caspase-cleaved p15 was determined by western-blotting. (**B**) The phosphorylation of Bad on Ser112 was measured by immunoblotting using a phospho-Bad Ser112 antibody after 30 min stimulation with TPA or PD 98059 + TPA. The relative induction (control = 1) was measured by densitometric measurement of the amount of Bad as visualized by an immunoblot using control Bad antibody to the amount of Bad phosphorylated on Ser112.

### *MAPK-mediated protection of CD95-induced apoptosis is not dependent on protein synthesis*

It is well established that many cell types resistant to CD95- or TNF-R1-mediated apoptosis can be sensitized

by pre-incubation with protein synthesis inhibitors such as CHX. Accordingly, it has been suggested that stimulation of CD95 and TNF-R1 can elicit a distinct protein synthesis-dependent protective signal (Wallach, 1997) and that viral as well as cellular proteins suppress CD95-mediated apoptosis (Tschopp *et al*., 1999). In particular, upregulation of c-FLIP (Irmler *et al*., 1997; Yeh *et al*., 1998) and Bcl-$x_L$ (Peter *et al*., 1997) during T cell activation has been indicated to suppress CD95-mediated apoptosis. In this respect, it has been suggested that MAPK suppresses CD95-mediated apoptosis through elevated expression of c-FLIP (Yeh *et al*., 1998). However, it should be noted that this study is based solely on RNA data and therefore does not reveal the possible effects on actual protein levels. Furthermore, a recent study indicates that the role of FLIP in protecting activated T cells is still unclear (Scaffidi *et al*., 1999a), which is in accordance with the present study, as we could not find a major role for FLIP. Apart from the protein inhibitor-based modulation of CD95 responses, there are reports demonstrating signals protecting from CD95-mediated apoptosis that occur post-translationally through phosphorylation-based signaling pathways, such as MAPK (Holmström *et al*., 1998, 1999; Ruiz-Ruiz *et al*., 1999; Wilson *et al*., 1999) and PI-3K (Häusler *et al*., 1998; Varadhachary *et al*., 1999). It is quite plausible that activation of MAPK would generate both a protein synthesis-dependent and -independent mechanism. In this scenario, both the protein synthesis-dependent and -independent effect would be important during different stages of T cell activation. This hypothesis is in accordance with a recent study showing both a protein synthesis-dependent and -independent MAPK-mediated protective effect of serum withdrawal-induced apoptosis in neurons (Bonni *et al*., 1999). The results presented in this study, showing that OKT3 suppresses CD95-mediated apoptosis in the presence of CHX, preclude the requirement for protein synthesis in the MAPK-mediated protection against CD95-induced apoptosis, which is in agreement with our previous studies (Holmström *et al*., 1998, 1999). Thus, a high constitutive MAPK activity seems to be sufficient for inhibition of the CD95-mediated apoptotic signal, which does not rule out additional protein synthesis-dependent protective mechanisms that could regulate CD95 responses during the later phase of T cell activation. Recently, FLIP has been shown to promote MAPK signaling following CD95 activation (Kataoka *et al*., 2000) in resistant T cells. It is tempting to speculate that the initial CD3-induced MAPK activation would tune FLIP into a MAPK-directed signaling mode.

### *MAPK activation does not affect formation of a functional DISC but suppresses overall cleavage of caspase-8 and Bid*

It is important to determine where in the CD95-mediated apoptotic signaling pathway MAPK is exerting its protective effect, in order to assess whether the cells still maintain their normal functions when CD95 is stimulated but the apoptotic response is not triggered. Our results show that MAPK activation does not suppress formation of a functional DISC. However, MAPK inhibits DISC-mediated activation of overall caspase-8 activity. This could take place by inhibition of caspase-8 processing, although the DISC is assembled, resulting in inhibition of both overall caspase activation and activation of the mitochondrial amplification loop through Bid. Interestingly, the PI-3K pathway has similarly been shown to restrict caspase-8 processing in CD95-resistant Th-2 cells, although the DISC is assembled (Varadhachary *et al*., 1999). Jurkat T cells are assumed to be type II cells in terms of the CD95 response, and in these cells caspase-8 is mainly activated after the mitochondrial amplification loop (Scaffidi *et al*., 1998). Therefore, the lack of caspase-8 activation hints at a defect in the mitochondrial amplification loop. In this respect, we found further evidence that MAPK acts upstream of mitochondria, as we observed that the cleavage of Bid by caspase-8 is inhibited in a MAPK-dependent manner, as well as the CD95-induced loss of the mitochondrial membrane potential (data not shown). Recently, it has been shown that Bid is a direct target of caspase-8 (Li *et al*., 1998; Luo *et al*., 1998) and that this cleavage can be inhibited by TPA treatment (Scaffidi *et al*., 1999b). Processed p15 Bid translocates to mitochondria causing release of cytochrome *c*. The assumption that MAPK activation inhibits the mitochondrial amplification loop in CD95-mediated apoptosis is further strengthened by data showing that TPA only inhibits CD95-mediated apoptosis in cells that require release of cytochrome *c* for the apoptotic response (type II cells), whereas TPA does not affect CD95-mediated apoptosis in cells where the apoptotic response depends solely on caspases (type I cells; Scaffidi *et al*., 1999b). The inhibition of apoptosis at the early steps in the apoptotic pathway is likely to be desirable, as only this way could cells escape from any harmful effects of a partially activated apoptotic effector machinery. This type of regulation would also be useful in situations where initiator caspase activity is required for signaling other than activation of apoptotic effector caspases. In fact, it has recently been shown that caspase activation would be required for normal T cell development (Kennedy *et al*., 1999), an observation that would warrant a direct regulatory mechanism of caspases that can be dynamically modified to enable direction of the signal according to a given situation.

### *Possible downstream targets of MAPK affecting CD95-mediated apoptosis*

An interesting cytosolic substrate for MAPK is Bad, a pro-apoptotic protein that functions in the regulation of the transmembrane potential in mitochondria. Bad is known to be phosphorylated by the PI-3K pathway on Ser112 and Ser136 (Datta *et al*., 1997; del Peso *et al*., 1997). Phosphorylation of Bad at these sites inhibits binding of Bad to Bcl-$x_L$ and induces binding of Bad to the 14-3-3 proteins, thereby inactivating Bad (Datta *et al*., 1997; del Peso *et al*., 1997) and freeing Bcl-$x_L$ and Bcl-2 to promote survival. Recent reports indicate that cell survival by the MAPK signaling pathway would be due to a similar mechanism. In these reports the MAPK pathway is demonstrated to phosphorylate Bad on Ser112, thereby dissociating it from Bcl-$x_L$ (Bonni *et al*., 1999; Scheid *et al*., 1999). Our results indicate a similar MAPK-dependent phosphorylation of Bad on Ser112, thus implying MAPK-mediated phosphorylation of Bad as a possible parallel mechanism for promoting cell survival. While there is no previous evidence of Bad being involved

in direct regulation of CD95 responses, its phosphorylation could add another level of cell survival promoting activity in T cells, i.e. one inhibitory signal is targeted directly at CD95 and another at the general mitochondrial activation mechanism. In comparison, it has been shown that the PI-3K pathway is able to phosphorylate and affect the activity of several proteins regulating the apoptotic process (Datta *et al.*, 1999).

While our study conclusively shows that CD95 responses are regulated by direct MAPK-mediated signaling, further characterization and understanding of the biological roles of phosphorylation-based signaling mechanisms in CD95 functions will be important in order to utilize these mechanisms for modulation of CD95 responses.

## Materials and methods

### Cell culture

The human leukemic T cell line, Jurkat (clone EG-1; ATCC, Manassas, VA), was cultured in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified incubator with 5% $CO_2$ in air at 37°C. The cells were kept at a density of 0.5–1.0 × $10^6$/ml. To test the effects of TCR activation, the cells were incubated at a density of 2 × $10^6$ with 100 ng/ml mouse anti-human CD95 IgM antibody (Kamiya Biomedical Company, Thousand Oaks, CA), or 1 μg/ml anti-mouse CD95 $IgG_3$ antibody (Jo2, PharMingen, San Diego, CA) for the indicated time periods, in the absence or presence of 100 μg/ml immobilized OKT3 (a kind gift from the R.W.Johnson Pharmaceutical Research Institute, Bassersdorf, Switzerland). OKT3 was immobilized by pre-incubating the wells with 100 μg/ml protein A (Sigma, St Louis, MO) in a 50 mM $Na_2HCO_3$/$NaH_2CO_3$ buffer pH 9.8 for 4 h in 37°C followed by incubation with 100 μg/ml OKT3 for 4 h at 37°C. The effect of MAPK activation was determined by pre-treating the cells with 20 nM TPA (Sigma), in the presence and absence of 100 μM CHX (Sigma), or by pre-treating the cells for 30 min with 30 μM PD 98059 (Calbiochem, La Jolla, CA) before CD95 stimulation.

Human peripheral T cells were prepared as described elsewhere (Klas *et al.*, 1993). Resting peripheral T cells were separated from whole blood using Hypaque medium, cultured in RPMI medium (10% FCS), at a density of 0.5–1.0 × $10^6$/ml, activated by addition of 1 μg/ml PHA (Sigma) for 24 h, and further stimulated 2 days later by addition of 50 U/ml interleukin-2 (Sigma).

### Analysis of DNA fragmentation and phosphatidylserine exposure

Detection of DNA fragmentation into oligonucleosomal DNA fragments by agarose gel electrophoresis was performed as described previously (Holmström *et al.*, 1998). To detect phosphatidylserine exposure by flow cytometry, Jurkat T cells and the primary peripheral T cells were washed once with phosphate-buffered saline (PBS) and incubated for 10 min on ice in 400 μl of binding buffer (2.5 mM HEPES–NaOH pH 7.4, 35 mM NaCl, 0.625 mM $CaCl_2$) with 1 μl annexin V–FITC (Alexis, Laufelfingen, Switzerland) and 10 μg/ml propidium iodide (Molecular Probes, Eugene, OR) and analyzed on a FACScan flow cytometer (Becton Dickinson, NJ).

### Analysis of CD95 expression

The effect of MAPK activation on CD95 expression on the cell surface was followed by fixing TPA-treated Jurkat T cells (15 and 30 min) with 3% paraformaldehyde, and incubating the cells in the presence of an anti-APO-1 antibody, followed by an FITC-conjugated secondary antibody (Zymed, San Francisco, CA) and detection by using a FACScan flow cytometer.

### Immunoblotting techniques

The levels of Bad, Bcl-2, Bcl-$x_L$ and FLIP were followed by immunoblotting the respective proteins after treatment with 100 μg/ml immobilized OKT3 for different times. Phosphorylation of Bad on Ser112 and cleavage of Bid were followed after the respective treatments that affected MAPK activity.

Immunoblotting was performed by lysing cells in Laemmli sample buffer and then resolving the proteins on 12.5% SDS–PAGE. The separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and probed with the specific antibody to Bad (Transduction Laboratories, Lexington, KY), phospho-Bad Ser112 (New England Biolabs, Boston, MA), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-$x_L$ (Transduction laboratories), Bid (Santa Cruz Biotechnology) and c-$FLIP_L$ (Alexis), respectively. The proteins were visualized by the ECL system (Amersham, Buckinghamshire, UK) after addition of appropriate HRP-conjugated secondary antibodies.

### MAPK activity assays

The MAPK assay was carried out as described previously (Holmström *et al.*, 1998). After immunoprecipitation of the kinase, it was incubated in the presence of 1 mg/ml myelin basic protein (MBP; Sigma) as substrate, using a kinase assay buffer with 25 μM ATP, 2.5 μCi of [γ-$^{32}$P]ATP (Amersham). The reaction was carried out for 15 min at 37°C and stopped by addition of 3× Laemmli sample buffer. The samples were resolved on 12.5% SDS–PAGE, and MBP phosphorylation was quantified with a phosphoimager (Bio-Rad Laboratories, Hercules, CA). In parallel with the immunocomplex kinase assays, we determined the amount of MAPK2 in the immunoprecipitates by using a rabbit anti-human MAPK1 and 2 antibody (New England Biolabs). After coupling to a secondary antibody (Zymed), the proteins were visualized with the ECL system.

### Transfection studies

Cells were transiently transfected by using DEAE dextran (Pharmacia LKB, Stockholm, Sweden) as described previously (Holmström *et al.*, 1998). Cells were allowed to rest for 48 h before the respective treatments. The DNA construct used was pMCL-HA-MKK1-K97M, encoding an HA-tagged dominant-negative form of the MKK1 (Mansour *et al.*, 1996), a kind gift from Natalie Ahn (University of Colorado, CO). For detection of transfected cells, the cells were collected by centrifugation, resuspended in PBS, and fixed on ice for 1 h with 3% formaldehyde in PBS. The cells were then washed with PBS and permeabilized with 0.2% NP-40 (Sigma) for 10 min at room temperature. Detection of the transfected cells was performed with 10 μg/ml monoclonal HA-specific antibody (12CA5, Boehringer Mannheim, Germany) followed by an FITC-conjugated anti-mouse secondary antibody and 10 mg/ml Hoechst (Molecular Probes). Cells were mounted in 50% glycerol and viewed under a Leica RMB epifluorescence microscope. Mock transfections were carried out using a pIRES-EGFP plasmid (Clontech Inc., Palo Alto, CA).

### Analysis of caspase-8 recruited to the DISC by western blotting

The amount of DISC-associated caspase-8 was determined as follows: $10^7$ cells were either treated with 2 μg/ml anti-APO-1 antibody for 5 min at 37°C and then lysed in lysis buffer [30 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), small peptide inhibitors 1% Triton X-100 (Sigma) and 10% glycerol] (stimulated condition) or lysed and then supplemented with anti-APO-1 (unstimulated condition). The CD95 DISC was then precipitated for 2 h at 4°C with protein A–Sepharose. After immunoprecipitation the beads were washed five times with 1 ml of lysis buffer. Caspase-8 was detected from the immunoprecipitates by western blotting with a caspase-8-specific antibody.

### In vitro translation and in vitro cleavage assay of caspase-8

Caspase-8/a was *in vitro* translated using a T7 polymerase-directed reticulate lysate system (TNT, Promega, Madison, WI). *In vitro* cleavage assays were performed as follows. CD95 DISC was immunoprecipitated from 5 × $10^7$ cells as described above. Subsequently, the beads (containing the DISC) were incubated in 50 μl of reaction buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CAPS, 10 mM dithiothreitol and 20% sucrose) for 24 h at 4°C with 0.5 μl of *in vitro* translated caspase-8/a. After boiling for 3 min in a standard reducing sample buffer the resulting products were analyzed on 15% SDS–PAGE with subsequent amplification (Amplify, Amersham Pharmacia Biotech), drying of the gels and autoradiography.

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