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Caspase Inhibition Blocks Human T Cell Proliferation by Suppressing Appropriate Regulation of IL-2, CD25, and Cell Cycle-Associated Proteins

Markus Falk,* Sandra Ussat,* Norbert Reiling,† Daniela Wesch,* Dieter Kabelitz,* and Sabine Adam-Klages2*8

Caspases have been described as proteases essential for the release of certain cytokines and for initiation as well as execution of apoptosis. Increasing evidence indicates, however, that caspase activity is also required for activation-induced proliferation of mature T lymphocytes. The molecular mechanism, how caspase activity facilitates T cell proliferation, is still controversially discussed. In this study, we show that proliferation of human T cells in response to a specific antigenic stimulus is completely prevented by caspase inhibition. In addition, we demonstrate that this lack of proliferation is due to a failure to initiate cell cycle progression, but not the result of increased T cell death. Our results demonstrate that caspase inhibition leads to strongly reduced IL-2 release, failure to up-regulate CD25, and a lack of proper regulation of cell cycle-associated proteins. Furthermore, T cell proliferation was partially rescued by addition of exogenous IL-2. Using Jurkat cells, we show that in the absence of caspase-8, the mitogen-induced activation of the transcription factor NF-κB is moderately diminished, while the activity of the composite element CD28 response element and NF-IL-2B AP-1 sites is strongly reduced. Finally, we provide evidence that caspase inhibition suppresses the activation of purified monocytes by bacterial Ags. The Journal of Immunology, 2004, 173: 5077–5085.

The protease family of caspases has two major functions: the generation of mature forms of distinct cytokines and initiation as well as execution of apoptosis in response to many cell death-inducing stimuli. During apoptosis, caspases are activated via an extrinsic pathway through triggering of death receptors (DR)3 or via an intrinsic pathway involving mitochondria (for review, see Ref. 1). In the past few years, however, it became apparent that caspases also play an important role for proliferation and differentiation of select cell types (for review, see Refs. 2–5). After the first observations that caspase-3 is activated in proliferating nonapoptotic T lymphocytes (6, 7), an increasing amount of in vitro data has demonstrated that caspase activity is essential for activation and proliferation of T cells (8, 9). These data were considerably strengthened by the observation that inactivation of caspase-8 in humans and mice leads to immunodeficiency due to a lack of functional T cells and, in the human system, B lymphocytes and NK cells (10, 11).

Interestingly, a well-known signaling pathway leading from the DR CD95 (Fas, APO-1) via the adapter protein Fas-associated death domain protein (FADD) to the activation of caspases had already been linked to the proliferative response of naive T cells (for review, see Ref. 12). In mice overexpressing a dominant-negative form of FADD or completely lacking functional FADD, not only apoptosis in response to DR stimulation, but also activation-induced proliferation of T cells, is inhibited (13–16). Additionally, the overexpression of dominant-negative FADD was found to restrict the proliferation of murine fibroblasts (17). DR signaling has previously been linked to growth stimulation in both T cells and fibroblasts. Stimulation of CD95 mediates a costimulatory signal in Ag-stimulated quiescent T cells and a growth-stimulatory signal in fibroblasts (9, 18, 19). Another death ligand, TNF, also stimulates the growth of T cells and fibroblasts (20, 21). Therefore, it is conceivable that the growth-stimulatory signal after DR ligation is generally dependent on caspase activity. The exact molecular mechanism, how caspases regulate cell cycle progression, is to date largely unknown.

A comparison of all of these reports, however, revealed important differences. In several studies, the defective proliferation in the absence of FADD/caspase signaling occurred despite normal secretion of IL-2 and could not be rescued by exogenous IL-2 (11, 13, 16). In addition, the reduction in the numbers of functional T cells in vivo was attributed to a high rate of cell death (11, 22). In contrast, in human T cells lacking caspase activity, the growth inhibition appeared to be due to a lack of IL-2 release (9, 10). One plausible explanation could be that these discrepancies are simply due to the use of different species. Another possibility would be that FADD and caspase-8 act independently during T cell proliferation. All studies investigating early responses to TCR stimulation showed that these early events occur unaltered in the absence of FADD/caspase signaling (11, 17, 23, 24). A failure to properly regulate cell cycle-associated proteins has been reported in FADD-deficient murine T cells (25).

In this study, we intended to define the molecular mechanisms by which caspases promote human T cell proliferation. The above described discrepancies made it necessary to first repeat some key experiments with human T cells. In this study, we demonstrate that...
in the response of caspase blockers, human T cells fail to proliferate in response to various stimulations due to a failure to induce the IL-2/IL-2R system. We provide evidence that the lack of functional caspase-8 leads to diminished transcriptional activity of NF-κB and especially of the composite element CD28 response element and NF-IL-2 AP-1 sites (RE/AP). Finally, our data indicate that caspases regulate monocyte function in antigenic stimulation.

Materials and Methods

Reagents and Abs

The broad-spectrum caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) and the cell-permeable caspase substrate benzyloxy carbonyl-Val-Ala-Asp-7-amido-4-methylcoumarin (zVAD.AMC) were purchased from Bachem (Weil am Rhein, Germany). Human rIL-2 was received from Chiron (Marburg, Germany), IL-1β from R&D Systems (Wiesbaden, Germany), and IL-18 from PeproTech (London, U.K.). PHA was obtained from Genzyme-Virotech (Rüsselsheim, Germany), purified protein derivative (PPD) and PMA from Sigma-Aldrich (Deisenhofen, Germany), and ionomycin from Merck Biosciences (Darmstadt, Germany). The mAb against anti-CD3 was provided by Orthoclone Janssen Cilag (Neuss, Germany), and the mAb against CD3 was provided by Orthoclone Janssen Cilag (Deisenhofen, Germany), and ionomycin from Merck Biosciences (Darmstadt, Germany). The purity of the T cells as determined by anti-CD3 staining and flow cytometry was at least 97%. For stimulation, cells were seeded at 5 × 10^5 cells/ml on plastic dishes coated with 20 μg/ml anti-CD3 mAb, in the case of purified T cells together with 2 μg/ml anti-CD28 mAb. For short-term stimulation, anti-CD3 and anti-CD28 mAbs were cross-linked using a rabbit anti-mouse antiserum. ZVAD.fmk and zVAD.AMC were used at a final concentration of 100 μM. PHA was used at a final concentration of 1 μg/ml and PPD at 10 μg/ml. PMA (10 ng/ml) in combination with ionomycin (500 ng/ml) was used as positive control. To quantify proliferation, 1 × 10^5 PBMC or purified T cells were stimulated in triplicates for 4 days in 96-well plates and labeled with 1 μCi/well [3H]thymidine (Amersham Biosciences, Freiburg, Germany) for 16 h. Cells were harvested, and DNA-associated radioactivity was counted by liquid scintillation.

Cell cycle analysis

Cell cycle analysis was performed, as previously described (26).

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**FIGURE 1.** Caspase inhibition blocks Ag-induced proliferation of primary T cells. A. Purified PBMC were cultured in medium alone or stimulated with immobilized anti-CD3 mAb in the absence or presence of 100 μM zVAD.fmk or with zVAD.fmk alone for 4 days. In parallel, purified T cells were stimulated with immobilized anti-CD3/CD28 mAbs in the absence or presence of 100 μM zVAD.fmk or with zVAD.fmk alone for 4 days. Proliferation was measured by [3H]thymidine incorporation, which is expressed as mean ± SEM of triplicate determinations. Results are representative of at least five different experiments. B. Purified PBMC were cultured, as described for A, and additionally stimulated with anti-CD3 mAb in the absence or presence of zVAD.AMC as negative control. After 4 days, proliferation was quantified by [3H]thymidine incorporation. C. Purified PBMC from tuberculin test-positive donor were cultured in medium alone or stimulated with PPD in the absence or presence of 100 μM zVAD.fmk. [3H]thymidine incorporation was determined after 4 days and is expressed as mean ± SEM of triplicate determinations. Results are representative of three independent experiments. D. Purified PBMC were cultured, as described for A, and cell cycle distribution was determined.
Determination of caspase activation

The activity of caspase-3 was determined using the cell-permeable fluorogenic substrate PhiPhiLux (Merck Biosciences), which was used according to the manufacturer’s instructions, and quantified by flow cytometry. Activated caspase-3 was detected by flow cytometry using a selective fluorogenic substrate (PhiPhiLux) in the living gate, as determined by forward light scatter/side light scatter. Purified T cells were cultured in medium or stimulated with immobilized anti-CD3/CD28 mAbs in the absence or presence of 100 μM zVAD.fmk for 4 days and lysed. From each lysate, 20 μg of protein was analyzed for the expression of different cell cycle-regulating proteins, as indicated.

Western blot analysis

Western blot analysis was performed, as previously described (26).

Measurement of IL-2 production

PBMC or purified T cells were stimulated, as described above, and stained for the expression of diverse activation markers on T lymphocytes using fluorescence-labeled Abs against CD3, CD69, CD25, and HLA-DR. T cells positive for the activation markers were quantified using the FACS Calibur Analyzer.
Biochemicals) using oligo-(dT) primer. The IL-2 cDNA was amplified using the primers 5'-AAC AGT GCA CCT ACT TCA AG-3' and 5'-GTT GAG ATG ATG CTT TGA CA-3' and the FastStart DNA Master SYBR Green kit in duplicates, followed by real-time PCR on a LightCycler (Roche Molecular Biochemicals). Standard curves were generated for each cDNA separately by RT-PCR of different dilutions with primers for the housekeeping gene GAPDH (5'-ACC ACA gTC gCT gCC AAC AC-3' and 5'-TCC ACC CTT TgT CGg TA-3'). Exact quantification was achieved by comparison of each standard curve with the respective PCR result for IL-2 using the LightCycler software (Roche Molecular Biochemicals).

**Luciferase assay**

A total of 1 x 10^7 wild-type or caspase-8-deficient Jurkat cells was transfected by electroporation (280 V, 960 fl) with 10 μg of reporter construct containing the firefly luciferase gene under the control of the IL-2 promoter (kindly provided by R. Abraham, The Burnham Institute, La Jolla, CA), several NF-κB or NF-AT binding sites (Stratagene, Heidelberg, Germany), or a composite RE/AP-binding sequence (kindly provided by A. Weiss, Howard Hughes Medical Institute, San Francisco, CA). After incubation for 24 h, equal aliquots of the cells were left untreated or stimulated with cross-linked anti-CD3/CD28 mAbs or PMA/ionomycin. After additional 5 h, cells were harvested, and luciferase activity was determined using the luciferase assay system, according to their instructions.

**Gel shift assays**

Gel shift assays to quantify activation-induced specific c-Rel binding were performed using the c-Rel Nushift assay kit from Active Motif (Rixensart, Belgium), according to the instructions of the supplier. For supershifts, 1 μl of a polyclonal c-Rel antiserum (Abcam, Cambridge, U.K.) was added after the binding reaction was completed.

**Bacterial stimulation of monocytes**

Monocytes were isolated from PBMC by counterflow centrifugation, as previously described (27). The purity of the monocyte fraction was determined using flow cytometric analysis of CD14 expression (consistently >95%) and positivity for α-naphthyl-esterase staining (>90%). Monocytes were activated with LPS (10 ng/ml) or infected with Mycobacterium avium strain SE01 (bacteria:monocytes, 5:1) either in the presence or absence of 30 μM zVAD.fmk. Supernatants were collected after 6 h, and TNF secretion was quantified by ELISA (H. Gallati, Intex, Muttenz, Switzerland) (28), according to the manufacturer’s instructions.

**Results**

**Caspase inhibitors block proliferation of human T cells**

It has been shown previously that the proliferation of resting human and murine T cells in response to anti-CD3 stimulation is impaired by the broad-spectrum caspase inhibitor zVAD.fmk (8, 9, 24). We isolated naive human T lymphocytes and verified that caspase inhibition interferes with T cell proliferation. The [3H]thymidine incorporation of human PBMC stimulated with plastic-bound anti-CD3 mAb was reduced in the presence of zVAD.fmk to a level below that seen in cells kept for 4 days in medium (Fig. 1A). No inhibition was detected using the structurally related caspase substrate zVAD.AMC (Fig. 1B). In purified T cells stimulated with anti-CD3/CD28 mAbs, the proliferation was also strongly inhibited by zVAD.fmk, although to a lesser extent than in PBMC (Fig. 1A). The lack of proliferation, however, was not due to increased cell death, as we never observed significantly more dead cells measured by propidium iodide staining after treatment with zVAD.fmk compared with cells stimulated with medium or anti-CD3 only (data not shown). Using increasing amounts of zVAD.fmk and of the selective inhibitor for caspase-8, zIETD.fmk, we observed a dose-dependent inhibition of T cell proliferation starting at a concentration of 10 μM, reaching a maximum at 50-100 μM (data not shown). The solvent used to solubilize the caspase blockers (ethanol or DMSO) had no growth-inhibitory effect on T cells, even when used in a 5-fold higher concentration (data not shown). To prove that zVAD.fmk also inhibits proliferation in response to a more physiological and specific Ag, we used PPD to stimulate PBMC from a donor tested positive in a tuberculin test. Again, proliferation of PBMC was completely inhibited by zVAD.fmk (Fig. 1C).

Salmena et al. (11) recently reported that the reduced activation-induced proliferation of murine T cells lacking caspase-8 is due to increased cell death, while cell cycle entry proceeds normally. Because we did not detect increased cell death after caspase inhibition, we analyzed the cell cycle distribution of PBMC stimulated

- **FIGURE 4.** IL-2 release is diminished by caspase inhibition. A, Purified PBMC were cultured in medium alone or stimulated with immobilized anti-CD3 mAb in the absence or presence of 100 μM zVAD.fmk or with zVAD.fmk alone for 24 or 48 h. Supernatants from duplicate cultures were collected and analyzed for secretion of IL-2 using ELISA. Indicated are the mean values from duplicate determinations. One of three independent experiments is shown. B, Purified PBMC were stimulated with PMA/ionomycin in the absence or presence of 100 μM zVAD.fmk for 24 or 48 h. IL-2 secretion was quantified in the supernatants from triplicate cultures using ELISA. The shown mean values ± SEM from triplicate determinations are representative for one of three independent experiments. C, Purified PBMC were cultured in medium alone or stimulated with immobilized anti-CD3 mAb in the absence or presence of 100 μM zVAD.fmk for 16 h. Total RNA was extracted and converted into cDNA, and IL-2 mRNA was amplified in duplicates by real-time PCR. The signal was quantified in comparison with the expression of GAPDH mRNA. Indicated are the mean values ± SEM from triplicate determinations. The results are representative for three independent experiments. D, Wild-type and caspase-8-deficient Jurkat cells were left untreated or stimulated with PMA/ionomycin for 24 and 48 h. Supernatants from triplicate cultures were collected and analyzed for secretion of IL-2 using ELISA. Indicated are the mean values ± SEM from triplicate determinations. One of three independent experiments is shown.
with anti-CD3 in the presence or absence of zVAD.fmk. As shown in Fig. 1D, anti-CD3 treatment drove a significant amount of lymphocytes to enter the cell cycle, which was completely abolished by zVAD.fmk.

Early reports had already indicated that caspases are activated in living T cells after anti-CD3 stimulation (6, 7). Therefore, we analyzed whether caspases are activated in PBMC in response to various mitogenic stimuli. PBMC were stimulated with anti-CD3 mAb or PHA for 4 days, and caspase-3 activity in the gated population of living lymphocytes was detected using the cell-permeable substrate PhiPhiLux G2D2 (Fig. 2A). This activity was strongly reduced, when the cells were stimulated in the presence of zVAD.fmk (data not shown). Next, caspase-3 activity was measured in purified T cells stimulated with anti-CD3/CD28 mAbs alone or in combination with zVAD.fmk. As shown in Fig. 2B, caspase-3 was active in the stimulated cells, but the activity was almost completely inhibited by zVAD.fmk. Activation of caspase-3 in healthy proliferating cells was confirmed by immunofluorescence staining of T cells stimulated with anti-CD3 mAb for 4 days with an antiserum against active caspase-3. Treatment with zVAD.fmk strongly inhibited this staining (Fig. 2C, a–c). Moreover, we show staining of PBMC stimulated with anti-CD3 Ab for 2 days with a mAb against active caspase-8, which again was inhibited by zVAD.fmk (Fig. 2C, d–f). Performing kinetic analyses with both Abs, we observed that activation of caspase-8 (maximal at day 2) clearly preceded that of caspase-3 (maximal at day 4; data not shown).

Caspase inhibitors suppress proper regulation of cell cycle-controlling proteins

Several cell cycle-regulating proteins have been reported as substrates for caspases (29). Blocking putative cleavage of a cell cycle-inhibiting protein would result in the unscheduled up-regulation of this protein, leading to cell cycle arrest. Therefore, the expression of cell cycle-regulating proteins in PBMC stimulated for 1–4 days with anti-CD3 mAb with or without caspase inhibition was analyzed. Although in proliferating T cells the expression of cyclin-dependent kinases, cyclins, and RB as well as its phosphorylation was up-regulated, zVAD.fmk largely prevented this up-regulation (Fig. 3). The expression of the cyclin-dependent kinase inhibitor p27kip1, which has been published as caspase substrate, decreased after anti-CD3 stimulation, and this down-regulation was inhibited by zVAD.fmk (Fig. 3). The specific cleavage product, however, was not observed in lysates from proliferating T cells even after very long exposures, while it was readily detected in apoptotic Jurkat cells (data not shown). These results indicate that caspase inhibition prevents proper regulation of all investigated cell cycle-associated proteins, thereby arguing for a more general mechanism preventing activation and proliferation than simply a lack of the removal of a cell cycle-inhibitory protein.

Caspase inhibition leads to a failure to activate the autocrine IL-2 system

Proliferation in response to TCR stimulation is facilitated by an autocrine activation loop involving the release of IL-2 and the up-regulation of CD25, the α-chain of the IL-2R (30). The influence of caspase inhibition on this autocrine system is controversially discussed. In this study, we demonstrate that caspase inhibition led to drastically reduced secretion of IL-2 in response to TCR stimulation in human PBMC (Fig. 4A) and purified T cells (data not shown). PMA/ionomycin-induced IL-2 secretion was still detected (Fig. 4B). The caspase substrate zVAD.fmk did not affect PMA/ionomycin-induced IL-2 secretion (data not shown). We next investigated whether the effect of caspase inhibition on IL-2 release occurs at the transcriptional level. Therefore, we analyzed the transcription of the IL-2 gene by quantitative RT-PCR. As shown in Fig. 4C, the amount of mRNA encoding IL-2 was strongly increased in PBMC after anti-CD3 stimulation, and this increase was almost completely inhibited by zVAD.fmk.

FIGURE 5. Exogenous IL-2 partially rescues the proliferation block induced by caspase inhibitors. A, Purified PBMC were stimulated, as described in Fig. 1. Additionally, 30 U/ml IL-2, 10 ng/ml IL-1β, or 10 ng/ml IL-18 was added to the cultures either alone, or in combination with anti-CD3 mAb, zVAD.fmk, or anti-CD3 + zVAD.fmk. After 4 days, [3H]thymidine incorporation was determined and is expressed as mean ± SEM of triplicate determinations, as indicated. Results are representative of three different experiments. B, Purified T cells were analyzed, as described for A, with the exception that both anti-CD3 and anti-CD28 mAbs were immobilized. Results from one of three independent experiments are shown.
Interestingly, PHA-induced IL-2 secretion of the leukemic T cell line Jurkat also requires caspase activity (31). A subclone of Jurkat cells has been described lacking functional caspase-8 (32), which was used to analyze the effect of caspase inhibition on IL-2 expression in Jurkat cells. Because the caspase-8-deficient cells display almost undetectable CD28 surface expression as determined by FACS analysis (data not shown), we used PMA/ionomycin as mitogenic stimulus. As shown in Fig. 4D, IL-2 secretion was induced after PMA/ionomycin stimulation in wild-type Jurkat cells, but strongly reduced in the caspase-8-deficient subclone.

Next, we investigated whether exogenous IL-2 overcomes the inhibitory effect of zVAD.fmk on T cell proliferation. Because caspase-1, which is also inhibited by zVAD.fmk, is essential for the release of IL-1β and IL-18 (1), we also tested these cytokines for a potential rescue effect. PBMC and purified T cells were stimulated with anti-CD3 mAb in the presence or absence of zVAD.fmk. IL-2 secretion was induced after PMA/ionomycin stimulation in wild-type Jurkat cells, but strongly reduced in the caspase-8-deficient subclone.

In addition to NF-κB, the transcriptional activities of NF-AT, AP-1, and the composite element RE/AP regulate the expression of the IL-2 gene (33). Moreover, NF-κB and NF-AT are also required for the activation-induced up-regulation of CD25 (34). Therefore, reporter constructs containing the luciferase gene driven by the IL-2 promoter or binding sequences specific for NF-κB, NF-AT, or RE/AP were used for transient transfection and reporter assays. Because Jurkat cells are easily transfectable, we chose to use these cells for the reporter assays. We first analyzed whether zVAD.fmk blocks the anti-CD3/CD28-induced luciferase expression in wild-type Jurkat cells. However, the addition of ethanol or DMSO alone, which were used to solubilize zVAD.fmk, already led to explicitly altered luciferase expression (data not shown). Therefore, we again made use of the caspase-8-deficient Jurkat subline and PMA/ionomycin as mitogenic stimulus. Wild-type and caspase-8-deficient cells were transfected with the respective reporter constructs and pooled. Because all stimulations were performed using the same pool of transfected cells, internal transfection controls were used to analyze the effect of caspase inhibition on the expression levels of other activation markers in human PBMC. Although the anti-CD3-induced up-regulation of the very early activation marker CD69 was only moderately reduced by zVAD.fmk, the up-regulation of CD25 was severely impaired (Fig. 6). A similar inhibitory effect of zVAD.fmk was detected on the up-regulation of HLA-DR (Fig. 6), CD95, CD154, and CD137 (data not shown). Taken together, our results suggest a major role of the failure to activate the IL-2/IL-2R system as cause of the observed defect in Ag-induced T cell proliferation in the presence of caspase inhibitors.

Transcriptional activity of NF-κB and the composite element RE/AP as well as c-Rel activation are diminished in caspase-8-deficient Jurkat cells

Our results indicate that caspase inhibition restricts one or more proximal signaling events necessary for the expression of growth-promoting proteins such as IL-2, CD25, and several cell cycle-regulating factors. Therefore, we analyzed the influence of caspase inhibition on early TCR-induced responses in human PBMC. The general pattern of proteins phosphorylated on tyrosine detected after anti-CD3 stimulation was not affected by zVAD.fmk (data not shown). The increase in the anti-CD3-induced activation of the protein serine kinases ERK1/2 and Akt, indicated by enhanced phosphorylation, was also not significantly inhibited by zVAD.fmk (data not shown). A slight, but reproducible decrease in IκB-α immunoreactivity indicative for activation of the transcription factor NF-κB was detected after anti-CD3 treatment for 90 min, which was less pronounced in the presence of the caspase blocker (data not shown).

In addition to NF-κB, the transcriptional activities of NF-AT, AP-1, and the composite element RE/AP regulate the expression of the IL-2 gene (33). Therefore, reporter constructs containing the luciferase gene driven by the IL-2 promoter or binding sequences specific for NF-κB, NF-AT, or RE/AP were used for transient transfection and reporter assays. Because Jurkat cells are easily transfectable, we chose to use these cells for the reporter assays. We first analyzed whether zVAD.fmk blocks the anti-CD3/CD28-induced luciferase expression in wild-type Jurkat cells. However, the addition of ethanol or DMSO alone, which were used to solubilize zVAD.fmk, already led to explicitly altered luciferase expression (data not shown). Therefore, we again made use of the caspase-8-deficient Jurkat subline and PMA/ionomycin as mitogenic stimulus. Wild-type and caspase-8-deficient cells were transfected with the respective reporter constructs and pooled. Because all stimulations were performed using the same pool of transfected cells, internal transfection controls were used to analyze the effect of caspase inhibition on the expression levels of other activation markers in human PBMC. Although the anti-CD3-induced up-regulation of the very early activation marker CD69 was only moderately reduced by zVAD.fmk, the up-regulation of CD25 was severely impaired (Fig. 6). A similar inhibitory effect of zVAD.fmk was detected on the up-regulation of HLA-DR (Fig. 6), CD95, CD154, and CD137 (data not shown). Taken together, our results suggest a major role of the failure to activate the IL-2/IL-2R system as cause of the observed defect in Ag-induced T cell proliferation in the presence of caspase inhibitors.

**FIGURE 6**. Expression of CD25 and HLA-DR, but not CD69, is attenuated by caspase inhibition. Purified PBMC were cultured in medium alone or stimulated with immobilized anti-CD3 mAb in the absence or presence of 100 μM zVAD.fmk or with PMA/ionomycin for 1–4 days. Cells were stained for the expression of CD25 and of the activation markers CD25, CD69, and HLA-DR, and analyzed by flow cytometry gated on lymphocytes. The mean fluorescence intensity of each activation marker within the CD3-positive population is indicated. The results shown are representative for three independent experiments.
such as Renilla luciferase construct for normalization were not necessary. In wild-type cells, the stimulation led to a strong induction of the IL-2 promoter-dependent luciferase activity, while the activity in caspase-8-deficient Jurkat cells was reduced by ~40% (Fig. 7). Stimulation with PMA/ionomycin also led to a strong activation of NF-κB, NF-AT, and RE/AP in wild-type cells. In caspase-8-deficient cells, the activation of NF-κB was moderately reduced, while NF-AT activation remained unaffected. The most prominent effect, however, was observed for the composite element RE/AP: the lack of caspase-8 led to a reduction in the transcriptional activity of RE/AP of ~80% compared with wild-type cells (Fig. 7). The transcriptional activity of the composite element RE/AP is regulated by possibly cooperative binding of the NF-κB family member c-Rel and a Jun/Fos heterodimer (35, 36). In mice, a disruption of the c-Rel gene alone is sufficient to eliminate IL-2 disruption (39). Therefore, we analyzed the activation of c-Rel in response to PMA/ionomycin stimulation in Jurkat cells using c-Rel-specific gel shift assays. In the absence of caspase-8, binding of a complex containing c-Rel was reduced compared with wild-type cells (Fig. 8A and B). The specificity of the gel shifts was confirmed using competition experiments with specific vs mutated oligonucleotides (Fig. 8A), and c-Rel binding was shown in supershifts using a c-Rel-specific Ab (Fig. 8C).

Taken together, our results indicate that caspase inhibition interferes with the activation of NF-κB and especially RE/AP, thus leading to reduced up-regulation of the IL-2/IL-2R system and, thereby, to deficient T cell proliferation.

**Activation of human monocytes is reduced by caspase inhibition**

Using human PBMC, zVAD.fmk blocked T cell proliferation below the level of cells treated with medium only, while the effect of caspase inhibition was less pronounced, when purified T cells were analyzed (Fig. 1). Therefore, we considered that caspase inhibition might have additional effects on APCs present in the PBMC preparations. Monocytes were purified from PBMC of healthy donors and stimulated with LPS, a known agonist for TLR-4, or infected with *M. avium* strain SE01, a TLR-2 agonist, either in the presence or absence of 30 μM zVAD.fmk. After 6 h, the release of TNF as indication for monocyte activation was determined. As shown in Fig. 9, the secretion of TNF after both stimuli was markedly reduced by caspase inhibition. Similarly, the up-regulation of CD14 was attenuated by zVAD.fmk, while the expression of HLA-DR was unchanged (data not shown). Significant cell death was not observed after up to 16-h incubation in the absence or presence of 30 μM zVAD.fmk (data not shown). Thus, the strong growth-inhibitory effect of zVAD.fmk observed in PBMC might partially be due to reduced monocyte activation.

**Discussion**

During the past years, it has been recognized that the role of caspases goes beyond the regulation of cytokine secretion and apoptosis. Unexpectedly, the proliferation of distinct cell types, most importantly T cells, requires caspase activity. This new function of caspases is completely independent from their role in apoptosis (for review, see Refs. 2–5). In this study, we provide evidence that, in human T cells, caspases are essential for the mitogen-induced activation of NF-κB and RE/AP, and, thus, for the induction of the IL-2/IL-2R system, and, thus, for the initiation of cell cycle progression. We cannot completely rule out the possibility that our observations solely result from the in vitro experimental settings used in our experiments. However, the fact that caspase-8 is essential for T cell expansion in vivo (10, 11) makes this possibility unlikely. In addition, our results show that caspase inhibition interferes with the activation of monocytes.

The requirement of the FADD/caspase signaling pathway for T cell proliferation was controversially discussed, until two reports were published showing that the lack of caspase-8 leads to defective T cell proliferation in vivo (10, 11). However, the molecular mechanism, how FADD/caspase signaling couples to T cell proliferation, appears to differ between mice and humans. Data derived from murine T cells have indicated that increased cell death mediates the inhibitory effect on T cell proliferation of blocking FADD/caspase signaling, while IL-2 secretion remains unchanged (11, 13, 22–24). Reports dealing with human T cells have favored that defective caspase activity inhibits cell cycle progression through the failure to activate the IL-2/IL-2R system (9, 10). The discussion about this discrepancy is still ongoing (37). Our data...
paired in caspase-8-deficient Jurkat cells. Protein kinase C-mediated activation of NF-/H9260 signaling pathways to transcriptional activation of IL-2 and CD25, will contribute to the activation of NF-AT (40), we could not detect involvement of caspase-8. The contribution for this apparent discrepancy is currently unknown. Although caspase-mediated cleavage (38) of the transcription factor c-Rel, which clearly corroborate the latter findings, as we have exclusively used human T cells.

The IL-2 gene expression in response to TCR stimulation is mainly achieved by the transcriptional activity of NF-κB, NF-AT, AP-1, and RE/AP (33). The expression of CD25 is, among others, also regulated by NF-κB and NF-AT (34). In this study, we have shown that the mitogen-induced activation of NF-κB and especially RE/AP is impaired in caspase-8-deficient Jurkat cells. Protein kinase C, which is essential for NF-κB activation in Ag-induced T cells, is activated by caspase-mediated cleavage (38). The transcription factor c-Rel, which is significantly involved in the activation of the RE/AP element, becomes inactivated upon caspase-mediated cleavage (29). Therefore, lack of caspase-8 could lead to enhanced binding of c-Rel to the RE/AP element, but not to the observed reduction. A possible explanation for this apparent discrepancy is currently unknown. Although caspase-mediated activation of the catalytic subunit of calcineurin can contribute to the activation of NF-AT (40), we could not detect impaired NF-AT activation in Jurkat cells lacking caspase-8. The detailed molecular mechanisms, how caspases are involved in the signaling pathways to transcriptional activation of IL-2 and CD25, will be subject of further studies.

Interestingly, the requirement of caspases for T cell proliferation appears to be limited to activation with strong antigenic stimuli, while weak TCR stimulation led to proliferation without the involvement of caspases (41). The logic behind this finding might be that a strong stimulus leads to massive T cell proliferation, which must be terminated fast and unmistakably. Therefore, the presence of preformed and activated caspases might be required to assure this fast termination of the immune response. Weaker stimulation of T cell proliferation on the other side, termination of the immune response might be less important. Alternatively, it has been suggested that the activation of caspases during T cell proliferation contributes to the development of a population of apoptosis-resistant memory T cells (42). However, the exact physiological function of the involvement of active caspases during T cell proliferation remains to be determined.

The presence of active caspase-3 in proliferating T cells opens up an important question: How are active caspases kept silent, until they are needed? Exact compartmentalization of active caspases, thus cleaving only a subset of substrates without inducing apoptosis, might be the answer. Such a subset of substrates could include cell cycle-inhibiting proteins, as already suggested (3). However, substantial cleavage of cell cycle-regulating proteins appears unlikely, because active caspases have been detected in living T cells by immuno-fluorescence exclusively in the cytoplasm (Fig. 2C, and our unpublished results). Along those lines, we have not observed the p23 cleavage product of p27KIP1 in proliferating T cells, although it was readily detected in apoptotic Jurkat cells. In addition, we have observed that caspase inhibition prevented the proper regulation of all investigated cell cycle-associated proteins induced by TCR stimulation. A severely defective regulation of select cell cycle-associated proteins, as has been seen in FADD-deficient resting and Ag-stimulated T cells (25), was not detected.

In murine T cells overexpressing dominant-negative FADD, a pronounced difference in the response of CD4+ T cells vs CD8+ T cells to anti-TCR stimulation was observed. Although the proliferation of CD4+ cells was only slightly reduced, the CD8+ cells showed a rather dramatic effect (22). Because we have not seen any significant proliferative response in the presence of the caspase blocker at all, it seems unlikely that the inhibitor had only a minor effect on the CD4+ cells in our system.

Additional evidence indicated that the activation of human monocytes in response to bacterial stimuli is impaired by caspase inhibition. Provided that zVAD.fmk also inhibited the activation of endogenous
monocytes within the PBMC preparations, this effect might explain the more pronounced inhibition of T cell proliferation in PBMC preparations compared with purified T cells. Because the broad-spectrum caspase inhibitor zVAD.fmk also blocks caspase-1, a lack of IL-1β release and subsequent signaling through the IL-1R might be the mechanism for the inhibitory effect of zVAD.fmk on cytokine release. Cytokine release of human monocytes in response to anti-Fas or LPS stimulation can be inhibited by caspase blockers. However, specific inhibition of IL-1β signaling did not prevent cytokine release (43). In addition, caspase inhibition can induce cell death in LPS-stimulated murine macrophages involving the orphan receptor Nur77 (44). We have also detected a significant amount of cell death after 16-h treatment with LPS in the presence of 100 μM zVAD.fmk (M. Falk and N. Reiling, unpublished results). However, using 30 μM zVAD.fmk, no significant cell death was detected. Thus, cell death is unlikely to be the reason for the reduced cytokine release.

Taken together, our results confirm previous studies reporting that active caspases are essential for T cell proliferation in response to antigenic stimulation. We provide additional evidence that in human T cells this nonapoptotic function involves a contribution to the activation of certain transcription factors needed for the induction of the IL-2/IL-2R system. Thus, caspase activity is essential for two obviously opposing responses, namely proliferation and apoptosis, in the same very cell type. Deciphering the exact molecular mechanisms, how caspases accomplish nonapoptotic functions, will further our understanding of the modulation between cell death and proliferation in response to TCR stimulation.

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References


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