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

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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 benzoyloxycarbonyl-Val-Ala-Aspfluoromethylketone (zVAD.fmk) and the cell-permeable caspase substrate benzoyloxycarbonyl-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 CD3 was provided by Orthoclone Janssen Cilag (Neuss, Germany), and the mAb against CD28 was purchased from Coulter Immunotech (Krefeld, Germany). FITC-labeled anti-CD3 Ab and PE-labeled anti-CD25, anti-CD69, and anti-HLA-DR were obtained from BD Biosciences (Heidelberg, Germany). Abs against cell cycle-regulating proteins were mostly purchased from BD Biosciences. Anti-cyclin A was obtained from Santa Cruz Biotechnology (Heidelberg, Germany), and anti-retinoblastoma protein (RB) and anti-phospho-RB from Cell Signaling Technology (Beverly, MA). Polyclonal antisera against active caspase-3 was obtained from Promega (Mannheim, Germany), and the mAb against active caspase-8 from Merck Biosciences.

Cell culture, stimulation, and proliferation assay

Wild-type and caspase-8-deficient Jurkat cells were kindly provided by J. Blenis (Harvard Medical School, Boston, MA) and grown in RPMI 1640 medium supplemented with 10% FCS and 50 μg/ml each of streptomycin and penicillin. PBMC were purified from buffy coats of healthy donors using Ficoll-Hypaque. T lymphocytes were purified from PBMC by magnetic separation using a negative selection kit from Dynal Biotech (Hamburg, 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 × 10^6 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[^H]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[^H]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[^H]thymidine incorporation. C, Purified PBMC from a tuberculin test-positive donor were cultured in medium alone or stimulated with PPD in the absence or presence of 100 μM zVAD.fmk.[^H]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. B, 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. C, Purified T cells (a–c) or purified PBMC (d–f) were left untreated (a and d), stimulated with anti-CD3 (b and e), or with anti-CD3 and 100 μM zVAD.fmk (c and f) for 2 days (d–f) or 4 days (a–c). Active caspase-3 (a–c) or active caspase-8 (d–f) was visualized by immunofluorescence staining.

Western blot analysis

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

Determination of activation markers by flow cytometry

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 FACSCalibur Analyzer.

Measurement of IL-2 production

PBMC were stimulated, as described above, for 1–4 days, and supernatants were obtained by centrifugation for 5 min at 2800 rpm. The IL-2 secreted by activated T cells was quantified using ELISA kits from R&D Systems or Bender Medsystems (Vienna, Austria), according to their instructions. Alternatively, the IL-2 transcription was quantified using real-time RT-PCR. PBMC or wild-type and caspase-8-deficient Jurkat cells were stimulated with immobilized anti-CD3 or anti-CD3/CD28 mAbs for 8–16 h. Total RNA was isolated using the High Pure RNA Isolation Kit, according to the instructions of the manufacturer (Roche Molecular Biochemicals, Penzberg, Germany), and cDNA was prepared with the First Strand cDNA Synthesis Kit for RT-PCR (avian myeloblastosis virus) (Roche Molecular Biochemicals).
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 CAT gCC ATC AC-3' and 5'-TCC ACC CNgT TGt TGt 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 μF) 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 SEH1 (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...
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 also clearly diminished by zVAD.fmk, although a significant release was still detected (Fig. 4B). The caspase substrate zVAD-AMC 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.
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. In parallel, IL-2, IL-1β, and IL-18 were added. After 4 days of stimulation, no enhancement of the anti-CD3-induced proliferation by exogenous IL-2 was detected, most likely due to the saturating concentration of the anti-CD3 mAb, while IL-2 by itself induced minor proliferation (Fig. 5A). Neither IL-1β nor IL-18 had any effect on basal or TCR-stimulated proliferation. As expected, zVAD.fmk completely inhibited the proliferation in response to anti-CD3 Ab. Only exogenous IL-2, but not IL-1β or IL-18, could partially revert the zVAD.fmk-mediated inhibition of T cell proliferation (Fig. 5A). Similar results were obtained using purified T cells (Fig. 5B, and data not shown). However, compared with the cells stimulated with anti-CD3 and IL-2 together, the proliferation of cells treated in addition with zVAD.fmk was still strongly reduced. Thus, the rescue effect of exogenous IL-2 was only moderate.

The surface localization of the high affinity IL-2R requires the expression of the IL-2R α-chain (CD25). CD25 is expressed on resting T cells at a low level, but is strongly induced after Ag stimulation. Therefore, we investigated whether zVAD.fmk also inhibited the up-regulation of CD25. In parallel, we analyzed the influence of the caspase blocker 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). 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 transf ectable, 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

**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 zVAD.fmk alone 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. 8, A 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 coupling 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

![Image](http://www.jimmunol.org/Downloadedfrom)
paired in caspase-8-deicient Jurkat cells. Protein kinase C/H9260 mitogen-induced activation of NF-

naling pathways to transcriptional activation of IL-2 and CD25, will

paired NF-AT activation in Jurkat cells lacking caspase-8. The de-
caspase-mediated activation of the catalytic subunit of calcineurin can

come 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 expla-
nation 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 im-
paired NF-AT activation in Jurkat cells lacking caspase-8. The de-
tailed molecular mechanisms, how caspases are involved in the sig-

naling 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 termi-
nated fast and unmistakably. Therefore, the presence of preformed
and activated caspases might be required to assure this fast termina-
tion of the immune response. After weaker stimulation of T cell pro-

liferation on the other side, termination of the immune response might
be less important. Alternatively, it has been suggested that the acti-
vation of caspases during T cell proliferation contributes to the de-
velopment of a population of apoptosis-resistant memory T cells (42).
However, the exact physiological function of the involvement of ac-
tive 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 immunofluo-
rescence 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 pro-
nounced 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 monocy-
ces in response to bacterial stimuli is impaired by caspase inhibition.
Provided that zVAD.fmk also inhibited the activation of endogenous

FIGURE 8. Activation-induced binding of c-Rel is reduced in caspase-8-deficient Jurkat cells. A, Wild-type and caspase-8-deficient Jurkat cells were left untreated or stimulated with PMA/ionomycin for 2 h, and nuclear extracts were prepared. Gel shift assays using an oligonucleotide containing a sequence allowing for c-Rel binding were performed. The same oligonucleotide and a mutated form thereof were used as competitors for specificity control. B, The results of six independent experiments, as described for A, were quantified by densitometric analysis. The binding activity detected in PMA/ionomycin-stimulated wild-type Jurkat cells was set to 100%. The mean value and the respective SD of the binding activity determined in PMA/ionomycin-stimulated caspase-8-deficient Jurkat cells are shown. C, Nuclear extracts from PMA/ ionomycin-stimulated wild-type and caspase-8-deficient Jurkat cells were used for c-Rel supershifts.

FIGURE 9. Caspase inhibition attenuates TNF release of human monocytes in response to bacterial stimulation. Highly purified human monocytes were stimulated with LPS or infected with M. avium strain SE01, either in the absence or presence of 30 μM zVAD.fmk. After 6 h, the secretion of TNF was determined in the supernatants using ELISA. Results are expressed as mean of duplicate determinations. One of two independent experiments is shown.
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 very same 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