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Cyclosporin A Abolishes CD28-Mediated Resistance to CD95-Induced Apoptosis via Superinduction of Caspase-3

Andreas Kerstan,*† Nicole Armbruster,* Martin Leverkus,†‡ and Thomas Hüning2*

Costimulation of T cells via CD28 promotes both proliferation and resistance to apoptosis. In this study, we show that the immunosuppressive drug cyclosporin A (CsA) fully reverses resistance to CD95-mediated cell death after TCR/CD28 costimulation or superagonistic anti-CD28 mAb stimulation of primary rat lymph node T cells. This effect correlated with a pronounced superinduction of caspase-3 on both mRNA and protein levels, whereas its main antagonist, X chromosome-linked inhibitor of apoptosis, was unaffected by inclusion of CsA. Apoptosis triggered by CD95 cross-linking was characterized by robust caspase-3 activation. Furthermore, CsA sensitization to CD95-mediated apoptosis of CD28-activated T cells did not alter mRNA stability of superinduced caspase-3 mRNA, suggesting a transcriptional regulation of the caspase-3 gene. Addition of Ca2+ ionophores to TCR/CD28 or superagonistic CD28-stimulated cells reduced caspase-3 levels, further supporting a role for Ca2+-dependent signaling pathways in negatively regulating caspase-3. Taken together, these findings suggest that CsA promotes sensitivity to CD95-mediated apoptosis in CD28-stimulated T cells by superinduction of the caspase-3 gene via a mechanism involving suppression of the calcineurin pathway. The Journal of Immunology, 2006, 177: 7689–7697.

The T cell response to Ag requires costimulatory signals that promote efficient T cell expansion and cytokine secretion. Transduction of costimulatory signals by the CD28 receptor is a pivotal element in the full stimulation of naive T cells, leading to enhanced cell survival and protection from activation-induced cell death (AICD)1 (1–3). In this respect, CD28-dependent signals are required for effective in vivo T cell responses to allografts (4, 5).

AICD, an important mechanism in the termination of immune responses, is primarily mediated via the CD95 death signaling pathway. Whereas restimulation of activated T cells through the TCR promotes AICD via the up-regulation of the CD95 ligand (CD95L) and concomitant acquisition of susceptibility to CD95-mediated apoptosis (6–9), CD28 costimulation counteracts AICD by simultaneously suppressing the TCR-mediated up-regulation of the CD95L (10) and increasing the expression of antiapoptotic molecules (11–14).

The CD95 signaling pathway is characterized by a sequential activation of caspases (15, 16). Activation of the effector caspase-3 represents one of the key points in the transmission of the CD95 death signal, leading to the biochemical and morphological changes that underlie apoptosis (17–19). Moreover, active caspase-3 amplifies the apoptotic stimulus via a positive feedback loop through autocatalytic activation and cleavage of other caspases, including caspase-8 (20, 21). The X chromosome-linked inhibitor of apoptosis (XIAP) represents one of the most potent caspase-3 inhibitors within the inhibitor of apoptosis family, acting not only through direct inhibition of caspase-3 enzymatic activity on its downstream targets (22, 23), but also through interference with autoproteolytic maturation of caspase-3 (24).

Calcineurin inhibitors such as cyclosporin A (CsA) have been proven to be effective in the treatment of a variety of T cell-mediated diseases, including allograft rejection. The immunosuppressive activity of CsA is largely ascribed to its ability to interfere with the Ca2+-dependent activation of NF-AT transcription factors. However, CsA has also been documented to promote CD95-induced apoptosis. Such an effect was first described by Kishimoto and Sprent (25), who demonstrated that resistance of TCR/CD28-costimulated murine CD4+ T cells to cell death by subsequent CD95 ligation is abrogated by addition of CsA during initial culture. The mechanism underlying this important finding has, however, remained elusive.

In this study, we address the question of this mechanism in a system that allows varying the contribution of TCR- and CD28-derived signals to the induction of T cell proliferation and acquisition of sensitivity to AICD (26). Primary T cells were either stimulated by mAb to the TCR alone, costimulated by inclusion of a conventional CD28-specific mAb, or directly activated by a superagonistic CD28-specific mAb that bypasses the requirement for TCR ligation. Although costimulation and, to a higher degree, CD28-driven T cell activation without TCR ligation induce resistance to CD95-mediated apoptosis, inclusion of CsA during T cell activation renders both costimulated and CD28 superagonist-activated cells highly susceptible to CD95-induced cell death. Under such conditions, caspase-3 is superinduced at both the mRNA and protein levels. Upon CD95 cross-linking, the up-regulated caspase-3 is rapidly activated. These studies support the notion that deletion of alloreactive T cell clones via CD95 might contribute to the immunological tolerance observed in transplantation medicine.
after CsA treatment, and provide a mechanistic basis for this concept.

**Materials and Methods**

**Abs and reagents**

mAb to rat αβ TCR (R73, IgG1), conventional (J319, IgG1), and mito-
genic (superagonistic, J316, IgG1) mAb to rat CD28 have been previously described (27). Polyclonal Abs to β-actin (C-11) were from Santa Cruz Biotechnology; polyclonal Abs to caspase-3 (9662) from Cell Signaling Technology; and polyclonal Abs to XIAP (AF822) from R&D Systems. For immunoprecipitation of XIAP, a mAb (AAM-050E) from StressGen Biotechnologies was used. Sheep anti-mouse IgG was from Boehringer Mannheim, and goat anti-mouse IgG peroxidase as well as goat anti-rabbit IgG peroxidase were from Dianova. CsA, actinomycin D, iodomyocin, as well as rat IL-2 were from Sigma-Aldrich. Ficoll was obtained from Am-

ersham Biosiences.

**Stimulation of rat T cells**

Freshly isolated lymph node T cells from 6- to 8-wk-old LEW rats kept under pathogen-free conditions were obtained by nylon wool passage. In all experiments performed, purity of T cells was ≥95% and cells were stimulated at a cell density of 7.5 × 10^5 cells/ml in supplemented X-VIVO 15 medium (Cambrex Bio Science). Experiments shown in Figs. 1, 2, 3, 7A, and 7B were performed in 96-well plates (Greiner Biosience), whereas 9-cm plastic dishes were used in Figs. 4, 5C, and 6, and 6-well plates in Figs. 5A, 5B, and 7C. Before stimulation experiments shown in Figs. 1C, 2, 3, 7A, and 7B, dead T cells detectable after nylon wool passage were removed by a Ficoll gradient. For TCR and TCR/CD28 costimulation, cells were incubated on plastic dishes precoated with sheep anti-mouse IgG, as described (28), followed by 2 μg/ml anti-TCR mAb (R73), without or with 0.5 μg/ml soluble conven-
tional anti-CD28 mAb (J319). For stimulation with anti-CD28 Abs alone, cells were cultured on sheep anti-mouse IgG-coated plates in the presence of 5 μg/ml soluble superagonistic (J316) or conventional (J319) anti-CD28 mAb. Proliferation was determined by pulsing triplicate cultures with [3H]thymidine (0.5 μCi/ml; Amershams Biosiences) 6 h before harvesting.

**Induction, inhibition, and detection of apoptosis**

Sensitivity to CD95-mediated apoptosis was assessed after the addition of a soluble CD95L, Flag-tagged fusion protein (0.1 μg/ml; Alexiss), which was cross-linked via an anti-Flag mAb (1 μg/ml; Enhancer; Alexiss) for the last 6 h of culture. Cells were harvested and resuspended in annexin V- 

**binding buffer (0.01 M HEPES (pH 7.4), 0.14 M NaCl, and 2.5 mM CaCl_2) containing FITC-labeled annexin V (BD Pharmingen) and 7-aminoactinomysin D (Sigma-Aldrich). After 15 min at 4°C in the dark, samples were diluted in buffer and immediately analyzed in a FACSCalibur flow cytometer (BD Biosciences).

**IL-2 ELISA**

IL-2 was detected using the OptEIA rat IL-2 set from BD Pharmingen, according to the manufacturer’s instructions.

**Western blot and immunoprecipitation**

Cells were washed twice in PBS and resuspended in whole cell lysis buffer (20 mM HEPES (pH 7.4), 2 mM EGTA (pH 7.9), 50 mM β-glycerophosphate, 2% SDS, 10% glycerol, 50 mM NaF, 0.01% NaN_3, 1 mM EDTA, 1 mM Na_3VO_4, 2 μM leupeptin, and 1 mM Pefabloc). Cells were kept on ice for 15 min and centrifuged at 12,000 × g for 20 min at 4°C. Lysates from 10^6 cells per lane were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (Hybond; Amersham), and subjected to immuno-

**blotting using the indicated Abs. For immunoprecipitation, 1 ml of 2% Nonidet P-40 lysis buffer (25 μM Tris (pH 7.5), 140 mM NaCl, 2 mM EDTA, 1 mM Pefabloc, 5 mM iodoacetamide, 1 mM Na_3VO_4, and 1 mM NaF) was added to 100 μl of cell suspension containing 5 × 10^5 cells. Lysates were centrifuged (12,000 × g, 10 min, 4°C) and the supernatant was added to protein G-Sepharose precoated with 5 μg of precipitating mAb to XIAP. Beads were incubated with rotation for 2 h at 4°C, followed by four washing cycles with lysis buffer before addition of 50 μl of SDS-

**PAGE sample buffer and electrophoresis (2 × 10^6 cells/lane). Proteins were transferred to nitrocellulose membranes, sequentially probed with polyclonal Abs (pAbs) to caspase-3 and XIAP, as indicated, followed by the appropriate secondary Ab-peroxidase conjugate, and developed using the ECL detection system (Amersham Biosiences).

**Detection of in vitro caspase-3-like protease activity**

To detect caspase-3-like protease activity, 2 × 10^6 cells were lysed using 40 μl of ice-cold CLB buffer (50 mM HEPES-KOH (pH 7.4), 0.1 mM EDTA, 100 mM NaCl, 0.1% CHAPS, and 1 mM DTT) and cleared by centrifugation (10,000 × g, 10 min, 4°C), and 10 μl of the supernatant was added to 50 μl of assay buffer (50 mM HEPES-KOH (pH 7.4), 0.1 mM EDTA, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, and 10% glycerol). After equilibration to room temperature for 10 min, the enzymatic reaction was initiated by addition of 200 μM Ac-DEVD-pNA (N-Acetyl-Asp-Glu-

Val-Asp-p-nitroaniline; Calbiochem), and cleavage of the substrate was monitored at 405 nm on a microplate reader (1420 Victor Multilabel Counter; Wallac). Experiments were set up in duplicates.

**Detection of DEVDase activity**

A total of 2 × 10^6 cells was lysed following treatment in buffer containing 50 mM HEPES-KOH (pH 7.0), 2 mM EDTA, 10% sucrose, 0.1% CHAPS, and 5 mM DTT. Equal amounts of protein were incubated with 1 μM biotinylated DEVD-acycloxyethyl ketone (biotin-DEVD-aomk, provided by D. W. Nicholson, Merck Frosst, Quebec, Canada) for 30 min at 37°C (24, 29). Biotin-DEVD-aomk is an irreversible caspase inhibitor that co-
vallently binds to DEVD-cleaving caspases, resulting in the biotin labeling of active caspase fragments (30). Samples were separated by 12% SDS-

**PAGE, and transferred to nitrocellulose membranes that were blocked overnight with 5% BSA. Following incubation with peroxidase-conjugated streptavidin (DAKO Diagnostics) for 1 h, biotin-labeled fragments were visualized with the ECL detection system (Amershams Biosiences). Subse-

quently, membranes were stripped and reprobed with polyclonal rabbit Abs to caspase-3 to detect caspase-3-specific fragments.

**RNase protection assay**

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technolo-

gies), and 5 μg was processed using BD Pharmingen’s RNase protection assay system (rat APO-1), according to the manufacturer’s instructions. Image data were collected with a phosphor imager (Fuji Photo).

**Statistical analyses**

Where indicated, data were subjected to Mann-Whitney rank sum tests (GraphPad Prism 3.0; GraphPad). Values of p < 0.05 were considered to be significant; p values of <0.01 were considered to be highly significant.

**Results**

CD28-driven proliferation is CsA resistant

As compared with proliferation induced by TCR stimulation alone, proliferation induced by TCR/CD28 costimulation is relatively insen-

sitive to the immunosuppressive drug CsA (31–36). To evaluate the impact of CsA on proliferation in our experimental system, we initially compared the proliferative response of freshly isolated rat lymph node T cells after TCR stimulation, TCR/CD28 costimu-

lation, and activation with superagonistic or conventional CD28-

specific mAb in the presence or absence of various CsA concentra-

tions. In line with our earlier results (27), proliferation induced by TCR/CD28 costimulation and superagonistic CD28 stimulation was comparable as assessed by [3H]thymidine incorporation, whereas TCR-stimulated cells proliferated poorly, and the conven-
tional CD28-specific mAb did not induce any measurable prolifer-

ation (Fig. 1A). The small proliferative response induced by TCR stimulation alone was reduced at low (33 ng/ml) and intermediate (100 ng/ml) concentrations of CsA, and was completely abolished at the highest concentration (300 ng/ml) used (Fig. 1B). Costimu-

lation via CD28, and to a greater extent activation by the super-

agonistic CD28-specific mAb, was less sensitive to inhibition by CsA. At a concentration of 100 ng/ml CsA, the proliferative re-

sponses of TCR/CD28-costimulated and superagonistic CD28 mAb-stimulated T cells were only slightly reduced, allowing a similar level of proliferation (Fig. 1B). Therefore, this concentra-

tion was used for further analysis of the influence of CsA on the acquisition of susceptibility to CD95-mediated cell death of TCR-

and CD28-triggered cellular responses. In agreement with reports
CD28 (co)stimulation in the presence of CsA primes T cells for CD95-mediated apoptosis

Next, we evaluated the influence of CsA on the development of sensitivity to CD95-induced apoptosis. T cells were stimulated for 24 h in the presence or absence of CsA, in agreement with reports by others (25, 33). This effect was significantly lower in line with the findings of Kishimoto and Sprent (25), because it could be completely blocked by a neutralizing anti-CD95L mAb.

Moreover, artificial CD95 cross-linking of TCR-stimulated cells revealed their high sensitivity to CD95-mediated cell death, in line with reports by others (32). IL-2 production was abolished under these conditions (Fig. 1C).

Lack of IL-2 secretion does not account for sensitization to CD95-induced apoptosis in CsA-treated, CD28-(co)stimulated cells

A well-known effect of the immunosuppressive activity of CsA is the inhibition of IL-2 production following TCR/CD28 stimulation (34, 38). The pleiotropic effects of IL-2 on cell survival are illustrated by the findings that whereas withdrawal of IL-2 from cells growing in an IL-2-dependent manner can initiate apoptosis, exposure to IL-2 actually promotes sensitization to CD95-mediated cell death (38, 39). Therefore, we tested whether exogenous IL-2 can rescue CsA-treated, TCR/CD28-costimulated, and superagonistic anti-CD28 mAb-activated cells from acquiring the observed CD95-sensitive phenotype. To this end, IL-2 was initially added to TCR/CD28-costimulated and superagonistic anti-CD28 mAb-activated T cell cultures. Fig. 2B illustrates that viability after CD95 triggering of CsA-treated, CD28-activated cells was largely independent of the presence of exogenous IL-2, excluding the possibility that lack of this cytokine is responsible for CsA-mediated acquisition of CD95 sensitivity.

Sensitization to CD95-mediated cell death requires the presence of CsA early after T cell activation

To evaluate the phase of T cell activation and clonal expansion during which CsA confers sensitivity to CD95-mediated apoptosis, TCR/CD28-costimulated and superagonistic anti-CD28 mAb-activated T cell cultures were subjected to different incubation periods with CsA. T cells undergoing TCR/CD28 costimulation or superagonistic CD28 stimulation were either continuously incubated with CsA for a time period of 24 and 48 h, or exposed to CsA after various periods of culture without the drug. Susceptibility to CD95-mediated cell death was assessed by addition of soluble CD95L, for the last 6 h of culture. As shown in Fig. 3, long-term T cell stimulation via TCR/CD28 or via CD28 superagonists in the presence of CsA for 24 and 48 h resulted in a pronounced sensitivity to CD95-induced cell death as compared with activation in the absence of CsA. Interestingly, when CsA was added during TCR/CD28 and superagonistic anti-CD28 mAb activation after 2, 4, or 17.5 h of culture time, a steady decline of apoptotic cells following CD95 ligation was noted. When CsA was added at 17.5 h, the percentage of apoptotic cells observed after CD95 ligation at 24 h was virtually indistinguishable from cultures stimulated in the absence of CsA. This effect was not due to the short
CsA incubation time, but rather to the long preactivation of T cells in its absence, because sensitivity to CD95-mediated cell death after an activation period for 24 h without CsA, followed by the addition of CsA for another 24 h, was also diminished to levels that were comparable to that observed without CsA (Fig. 3).

These findings indicate that CsA-dependent sensitization to CD95-mediated apoptosis of CD28-(co)stimulated cells requires the addition of CsA at the onset of culture, excluding a direct effect of CsA treatment on the CD95 signaling pathway.

**Superinduction of caspase-3 mRNA in CD28-(co)stimulated cells in the presence of CsA**

One explanation for the ability of CsA to overcome CD95 resistance could be its influence on the levels of pro- and antiapoptotic molecules. To evaluate this possibility, the mRNA levels of a panel of apoptosis-related proteins were assessed by RNase protection analysis. In agreement with reports by others (37), inclusion of CsA to CD28-(co)stimulated T cells did not alter the expression of CD95, excluding the possibility that enhanced expression of the death receptor is responsible for the observed sensitization to CD95-induced cell death (Fig. 4). TCR/CD28 costimulation and direct CD28 stimulation resulted in a moderate increase in caspase-3 mRNA expression after 24 and 48 h as compared with unstimulated or TCR-only-stimulated cells. However, in the presence of CsA, caspase-3 mRNA levels were dramatically increased (Fig. 4), suggesting that the apoptosis-sensitizing effect of CsA may be mediated through this caspase.

**Rapid caspase-3 activation after CD95 triggering in CsA-treated, CD28-(co)stimulated cells**

Caspase-3 is the major effector caspase promoting cell death in most cell types (18). To investigate whether the increased caspase-3 mRNA levels we observed translated into enhanced caspase-3 activity in response to CD95 cross-linking, cell lysates were analyzed by monitoring hydrolysis of the caspase-3-like substrate Ac-DEVD-pNA. As shown in Fig. 5A, CD95 ligation of TCR/CD28-costimulated or CD28 superagonist-stimulated cells cultured in the absence of CsA induced a marginal increase in caspase-3 activity. Despite the caspase-3 superinduction at the mRNA level (Fig. 4) and in line with the cell survival data (Fig. 2), neither TCR/CD28-costimulated nor superagonistic CD28-activated cells cultured in the presence of CsA displayed spontaneously increased caspase-3-like enzymatic activity (Fig. 5A). However, after CD95 cross-linking, a robust induction of caspase-3 protease activity was observed. In contrast, TCR stimulation either in the presence or absence of CsA resulted in similarly low levels of caspase-3 activation in response to CD95 triggering.

Activation of caspase-3 begins with the proteolytic cleavage of the large and small subunits, followed by removal of the prodomain. Although the first step is mainly regulated via active initiator caspases such as caspase-8, the second step is mediated by auto-proteolytic maturation of caspase-3 (18). To further characterize the superinduction and activation of caspase-3 in CsA-treated
CD28-activated T cells at the protein level, we examined caspase-3 expression by Western blot as well as caspase-3 activity by biotinylation of catalytically active caspase-3 in cell lysates stimulated under the different conditions. The latter technique allows the simultaneous detection of enzymatic activity and m.w., enabling the parallel monitoring of cleavage and activity of caspase-3.

As shown in Fig. 5B, TCR/CD28-costimulated cells displayed very little of the cleaved p17 fragment of caspase-3. Following

FIGURE 5. Analysis of enzymatic activity, the zymogen’s processing and interaction with XIAP of caspase-3. A, Induction of caspase-3-like enzymatic activity after CD95 triggering. T cells were stimulated for 19 h with the indicated mAb either in the presence or absence of CsA (100 ng/ml). sCD95L was added for the last 1 h of culture, as indicated. After preparation of whole cell extracts, the hydrolysis of the colorimetric caspase-3 substrate, Ac-DEVD-pNA, was monitored at λ = 405 nm over assay time (min), as described in Materials and Methods. One representative of three independent experiments is shown. B, Full processing of superinduced caspase-3 to a 17-kDa fragment in CD95-triggered, CsA-treated CD28-(co)stimulated T cells. Cells were stimulated and treated with sCD95L, as in A. Cellular lysates were incubated with biotinylated DEVD-aomk, as described in Materials and Methods, followed by Western blotting and subsequent probing with streptavidin-HP (SP-HP) and pAbs to caspase-3. DEVDase-specific caspase activity of cleaved fragments (p17/p20, third panel from above) was only detectable in cells treated with sCD95L. Under neither condition, labeling of full-length caspase-3 was observed. Subsequent rehybridization with pAbs to caspase-3 together with long (30 s) as well as short (10 s) exposure to autoradiographs confirmed position of the p17/p20 fragments. In parallel, whole cell extracts prepared as described above were blotted and probed with pAbs to XIAP. β-actin serves as a loading control. Results show one representative of three independent experiments. C, Coimmunoprecipitation of caspase-3 with XIAP. TCR/CD28 costimulation and incubation with CsA and sCD95L were conducted, as in A. Following immunoprecipitation with mAbs to XIAP, processed (p17), but not full-length caspase-3 was detected. Sequential probing of membranes with pAbs to XIAP revealed pronounced loss of XIAP in CsA-treated, CD95-triggered T cells. Detection of the Ig H chain of the precipitating Ab confirms equal precipitation conditions. Results depict one representative of two independent experiments.

FIGURE 4. Superinduction of caspase-3 mRNA in CsA-treated, TCR/CD28-costimulated, and superagonistic anti-CD28 mAb-activated cells. Total RNA obtained from T cells stimulated with the indicated mAb for 24 or 48 h either without or with 100 ng/ml CsA (○) was analyzed by RNase protection. The protected fragments for CD95, Bcl-xL, CD95L, caspase-1, caspase-3, caspase-2, and Bax mRNAs or the control L32 mRNA are indicated. After normalizing mRNA expression levels against the L32 mRNA, caspase-3 mRNA expression in T cell cultures stimulated via TCR/CD28 costimulation or superagonistic anti-CD28 mAb activation in the presence of CsA was ~3.5- to 5-fold increased after 24 h and 4.5- to 6-fold increased after 48 h as compared with T cells stimulated in the absence of CsA. Values are representative of three independent experiments (data not shown).
short-term (1 h) CD95 triggering, an enhanced caspase-3 cleavage pattern, including the p20- and p17-kDa fragments, together with detectable enzymatic activity of the p17 fragment, was observed (Fig. 5B). In line with the mRNA data, inclusion of CsA resulted in a strong up-regulation of the caspase-3 protein. However, subsequent CD95 triggering of these cells led to a complete degradation of this superinduced full-length caspase-3, along with the appearance of large amounts of the cleaved and enzymatically active p17-kDa fragment, but only minor p20 amounts. Virtually the same results were obtained with cells directly activated by superagonistic anti-CD28 mAb.

The rapid and pronounced cleavage of caspase-3 along with its enhanced enzymatic activity following CD95 cross-linking in CsA-treated cells points to an autoproteolytic maturation of superinduced caspase-3. This notion is further supported by the finding that relative amounts of p17/p20 fragments are shifted toward p17 under these conditions, which becomes apparent in short exposures of autoradiographs. Because autocatalytic maturation of caspase-3 is counteracted by XIAP, the most potent inhibitor of effector caspases (22, 23, 40, 41), an imbalance in the ratio of caspase-3 and XIAP after caspase-3 superinduction could favor such accelerated processing. Intriguingly, XIAP was readily detected in CD28-(co)stimulated T cells, but, in contrast to caspase-3, was not up-regulated when CsA was included (Fig. 5B, lower panel). Importantly, following CD95 triggering, CsA-treated cells displayed a pronounced loss of full-length XIAP consistent with the rise in caspase-3 protease activity (Fig. 5, A and B, middle panel) and with the extent of apoptotic cell death (Fig. 2). Conversely, XIAP was not degraded and potentially inhibited active caspase-3 in CD95-treated CD28-(co)stimulated cells. Thus, the dramatic shift in the ratio of procaspase-3 to XIAP after CD28 (co)stimulation in the presence of CsA may tip the balance in favor of apoptosis upon CD95 triggering.

To further investigate the interaction of XIAP and active caspase-3 under these conditions, cell lysates of TCR/CD28-(co)-stimulated cells activated in either the absence or presence of CsA, followed by incubation with soluble CD95L (sCD95L) for the last 1 h of culture, were immunoprecipitated using mAb to XIAP. As expected, XIAP coimmunoprecipitated with active caspase-3 (p17) in TCR/CD28/(co)-stimulated cells. Increased levels of p17 after CD95 triggering (Fig. 5B) went along with increased binding of XIAP to this fragment (Fig. 5C), suggesting that XIAP is at least partially able to neutralize p17 in these apoptosis-resistant T cells. Strikingly, the low levels of XIAP precipitated in CsA-treated, TCR/CD28-(co)-stimulated cells after CD95 triggering bound p17 at least as efficiently as in the other groups, but the low abundance of XIAP as compared with the massive amounts of active p17 present in these cells (Fig. 5B) indicates that despite this interaction it could not effectively counteract caspase-3 activity. Because XIAP is a target for caspases, including caspase-3 (42), its degradation is likely to be mediated by the overwhelming amount of active caspase-3 itself. This notion is supported by the preservation of normal XIAP levels when CD95 triggering was conducted in the presence of a caspase-3-like protease inhibitor, z-DEVD-fmk (data not shown).

Taken together, our findings demonstrate that concomitant with the induction of apoptosis, CD95 triggering of CsA-treated TCR/CD28-(co)-stimulated as well as superagonistic anti-CD28 mAb-stimulated T cells induced a rapid processing of superinduced caspase-3 to its active 17-kDa fragment along with a pronounced degradation of XIAP. This correlation suggests that an exceptionally high level of procaspase-3 without stoichiometric changes in XIAP after CD28 (co)stimulation in the presence of CsA might be responsible for CsA-induced sensitization to CD95-mediated apoptosis.

Unaltered mRNA stability of superinduced caspase-3 mRNA in CsA-treated CD28-(co)stimulated T cells

To address the question of whether the superinduction of caspase-3 mRNA in CsA-treated, CD28 (co)stimulated cells is due to increased mRNA stability, we stimulated T cells via TCR/CD28 or superagonistic anti-CD28 mAb with or without CsA for 12 h, followed by the addition of actinomycin D for various time periods, and analyzed caspase-3 mRNA levels. Fig. 6A illustrates that caspase-3 mRNA was readily detectable 12 h after T cell stimulation with superagonistic CD28 mAb, and levels steadily decreased over a time period of 8 h after inhibiting mRNA synthesis by the inclusion of actinomycin D. Parallel experiments conducted with the initial addition of CsA to superagonistic CD28-activated T cell cultures confirmed the robust induction of caspase-3 mRNA and demonstrated that the decline of caspase-3 mRNA in T cells stimulated in the presence of CsA was virtually indistinguishable from the decline observed in T cells stimulated in its absence (Fig. 6B). Similar results were obtained when T cells were costimulated via TCR/CD28 (data not shown).

In conclusion, these results indicate that the superinduction of caspase-3 is likely to be due to enhanced transcription rates rather than posttranslational mechanisms. Future studies are required to directly address caspase-3 transcription in our model system.

Strong Ca$^{2+}$ signals reduce caspase-3 expression in CD28-(co)stimulated T cells

CsA interferes with the intracellular Ca$^{2+}$ signaling pathway through inhibition of the serine-threonine phosphatase calcineurin

![FIGURE 6. Unaltered mRNA stability of superinduced caspase-3 in CsA-treated, superagonistic anti-CD28 mAb-stimulated T cells. A. RNase protection assay. Total RNA of superagonistic anti-CD28 mAb-stimulated T cells in the absence or presence of CsA (100 ng/ml; £8) for 12 h, followed by incubation with actinomycin D (5 µg/ml) for an additional 1, 2, 4, and 8 h, was analyzed by RNase protection assays. The protected fragments for CD95, Bcl-xL, CD95L, caspase-3, and Bax mRNAs or the control L32 mRNA are indicated. Results shown are representative of two independent experiments. B. Equal turnover of caspase-3 mRNA in cells stimulated in the absence or presence of CsA. Relative mRNA expression levels of caspase-3 observed in A were normalized against the control L32 mRNA and are shown as percentage of maximum expression (100%) for each time point.](http://www.jimmunol.org/DownloadedFrom)
Given our present finding that caspase-3 expression in TCR/CD28-costimulated as well as CD28 superagonist-activated cells is most likely positively regulated by CsA at the transcriptional level, we hypothesized that, conversely, Ca²⁺ signals may negatively regulate the transcriptional activity of the caspase-3 gene. To test this hypothesis, T cells were stimulated via TCR/CD28 or superagonistic CD28 mAb either alone or in the presence of various concentrations of the Ca²⁺ ionophore, ionomycin. Because ionomycin is also toxic to T cells (45), we titrated the ionophore to find concentrations that did not affect the viability of CD28-(co)stimulated cells (Fig. 7A) and only marginally influenced proliferation (Fig. 7B). As shown in Fig. 7C, inclusion of 0.125 or 0.25 μM ionomycin to CD28-(co)stimulated cultures at these subtoxic concentrations clearly reduced the levels of procaspase-3 in these cells. This reduction was not due to enhanced processing of procaspase-3 because no cleaved caspase-3 fragments were detected (data not shown).

Taken together, these results suggest that expression of the caspase-3 gene in TCR/CD28-costimulated as well as superagonistic anti-CD28 mAb-activated T cells is negatively regulated by Ca²⁺-dependent signaling pathways. Conversely, the superinduction of caspase-3 in CD28-(co)stimulated cells in the presence of CsA (Fig. 3) is likely to be mediated through the inhibition of such Ca²⁺-dependent signaling pathways, most likely through inhibition of calcineurin activity by CsA.

### Discussion

The relative sensitivity of TCR-mediated T cell activation to suppression by CsA as compared with that induced by costimulation through CD28 has been taken as an indication of distinct signaling pathways initiated through the TCR vs CD28 (36, 46). In extension of previous reports (25, 37), we show in this study that these differential effects of CsA on T cells activated by TCR vs CD28 ligation extend to the acquisition of sensitivity to CD95-mediated apoptosis, which is not observed in T cells that are only stimulated through the TCR. Rather, the abrogation of resistance to CD95-mediated cell death by CsA depends on CD28-derived signals. The importance of CD28-derived signals for this effect is further stressed by the marked resistance of T cells stimulated with a CD28 superagonist to CD95-mediated cell death, and their dramatic sensitization to this pathway of apoptosis by exposure to CsA. It should be mentioned in this context that recent work from our group has shown that mitogenic signals triggered by CD28 superagonists are not fully separate from the TCR signaling cascade, apparently because they require tonic signals emanating from the unligated TCR with which they converge downstream of ZAP-70. Thus, whereas experiments with primary rat and human T cells clearly showed that CD28-mediated T cell activation is not associated with tyrosine phosphorylation of TCRζ, ZAP-70, and linker for activation of T cells beyond unstimulated backgrounds, indicating that the mitogenic mAb do not directly or indirectly address the TCR complex (28, 47, 48), we found that CD28 superagonist-induced activation of T cell hybridomas is strictly dependent on the presence of the TCR, and on the next two downstream players of TCR signaling, the Syk family kinase ZAP-70 and the transmembrane adaptor linker for activation of T cells (49) (K. Dennehy and T. Hüning, unpublished observations). This suggested that in contrast to our earlier assumptions, mitogenic CD28 signals are not truly autonomous, but depend on low-level spontaneous or tonic signals from the TCR complex. Such signals are indeed known to exist and to be required for the maintenance of T cell identity (50).

As the likely point of convergence of such tonic TCR signals with the CD28 superagonist-induced signaling cascade, we have recently identified the SLP-76 signalsome. In spite of the dependence of mitogenic CD28 signals on the presence of the TCR, qualitative differences in the responses induced by TCR triggering alone, TCR plus CD28 costimulation, and CD28 superagonists indicate that besides this common pathway, unique downstream events also exist. An example for this is the promotion of

![FIGURE 7. Strong Ca²⁺ signals reduce caspase-3 expression in TCR/CD28-costimulated and superagonistic anti-CD28 mAb-activated T cells. A. Apoptosis of T cells induced by ionomycin. T cells were incubated for 18 h either in the absence or presence of falling concentrations (μM) of ionomycin (see inset) with the indicated stimulating mAb. Percentage of apoptotic cells was determined, as described in Fig. 2. Data of four independent experiments performed in duplicates are shown (±SD). As compared with cultures without ionomycin, the frequency of apoptosis obtained in the presence of 0.25/0.125 μM ionomycin was significant only in unstimulated cultures (p < 0.01). B. Effect of ionomycin on proliferative responses. T cells were stimulated with the indicated stimulating mAb for 24 h without or with various concentrations of ionomycin, as in A. At 6 h before harvesting, cells were pulsed with [³H]thymidine. Data are expressed as the mean cpm of triplicate wells (±SD) obtained from four independent experiments. Data obtained from stimulated T cell cultures incubated with 0.25/0.125 μM ionomycin were not significant as compared with stimulated cultures only. C. Diminished caspase-3 expression of activated T cells in the presence of ionomycin. Cells were either left unstimulated or stimulated for 18 h either in the absence or presence of various concentrations of ionomycin, as indicated. Total cellular lysates of 5 × 10⁶ cells were analyzed for caspase-3 expression by Western blotting and probing with pAbs to caspase-3. β-actin serves as a loading control. Data are representative of three independent experiments.](http://www.jimmunol.org/Download)
Th2 differentiation and GATA-3 expression by CD28 superagonists (51).

Importantly, acquisition of CD95-mediated apoptosis by T cells after CD28-driven activation in the presence of CsA correlated with a dramatic superinduction of caspase-3, the prime executor of CD95-mediated apoptosis (17), but not of its main physiologic antagonist, XIAP. We favor the hypothesis that upon triggering of the death receptor CD95, activation of caspase-3 by upstream initiator caspases may thus become independent of the mitochondrial amplification loop (52), which in costimulated T cells is normally blocked by CD28-dependent up-regulation of Bcl-xL (11). Indeed, the rapid and massive activation of superinduced procaspase-3 upon CD95 triggering, as well as the protective effect of a caspase-3 inhibitor (DEVd-fmk; data not shown) suggest that up-regulation of caspase-3 might represent a key mechanism for sensitization to CD95-mediated cell death by CsA. However, our data do not formally exclude mitochondria as the causative factor of initiating the apoptotic process after CD95 challenge of CSA-treated, CD28-activated cells. Alternative mechanisms involved could be an increased Smac and/or HtrA2 release, thereby leading to increased inhibition of XIAP, which consecutively goes along with an enhanced caspase-3 activity (53).

Caspase-3 was dramatically up-regulated at both the mRNA and the protein levels in CSA-treated cultures. Because mRNA stability was unaffected by CsA, increased transcription of the caspase-3 gene is the likely cause for this effect. This stimulatory effect of CsA on caspase-3 transcription was unexpected because usually caspases are regulated at the level of (auto)proteolytic events, leading to maturation and activation (54). However, elevated caspase-3 expression levels are observed during neuronal apoptosis after cerebral ischemia (55, 56) and increased caspase-3 levels are found in Ag-specific effector T cells, where they directly influence sensitivity to AICD (57).

The molecular mechanism underlying the positive regulation of the caspase-3 gene in the presence of CsA remains unsolved. The observation that like CsA, FK506, another calcineurin-inhibiting, immunosuppressive drug, is also capable of sensitizing CD28-(co)stimulated T cells to CD95-mediated apoptosis, whereas rapamycin fails to do so (data not shown), points to calcineurin activity as the critical factor for negatively regulating the caspase-3 gene. This conclusion is supported by the ionomycin-induced suppression of caspase-3 expression. Which downstream factors of calcineurin might be responsible? The calcineurin-regulated transcription factor, NF-AT, negatively regulates the cyclin-dependent kinase 4 (CDK4) through recruitment of histone deacetylase activity, 1, thus inhibiting the positive regulation of the CDK4 promoter through E2F (58). Because the rat caspase-3 promoter (59) contains two putative E2F binding sites immediately 5’ of a putative NF-AT binding site (our personal unpublished observation), it is tempting to speculate that such a NF-AT-mediated suppression as observed for the CDK4 gene is also operative for caspase-3. In support of this concept, inhibition of calcineurin through CsA/FK506 boosts transcription of the CDK4 gene (58) as it is the case for the caspase-3 gene. Studies are currently underway to test this hypothesis.

CsA-mediated sensitization to CD95 triggering may provide a mechanistic basis for the tolerance-promoting effect of CsA treatment during the acute allograft response (60–63). In line with this notion is the observation made in a model of unilateral lung allotransplantation, in which liposome-mediated CD95L transduction in combination with CsA led to an increase in apoptotic cells within the donor lung and to reduction of acute rejection (64). However, the apoptotic cell type and the efficiency of CD95L gene transfer in this system remain to be elucidated.

In summary, our studies demonstrate a novel effect of CsA treatment, which leads to the up-regulation of caspase-3 in T cells fully activated by CD28-derived signals. This up-regulation of caspase-3 might play the decisive role in breaking apoptosis resistance to CD95-mediated cell death usually conferred by TCR/CD80 costimulation. CsA-induced sensitivity to CD95-mediated cell death may thus contribute to immunological tolerance achieved by CsA treatment via deletion of reactive T cell clones.

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Disclosures

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