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Activation-induced cell death in murine T cell hybridomas. Differential regulation of Fas (CD95) versus Fas ligand expression by cyclosporin A and FK506

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Abstract

We have previously reported that activation of murine T cell hybridomas leads to expression of Fas (CD95) and its ligand (FasL) which subsequently interact, even on the same cell, leading to apoptotic cell death. Since the immunosuppressive drugs cyclosporin A (CsA) and FK506 block activation-induced apoptosis in T cell hybridomas, we examined whether such compounds affect cell death by interfering with expression of Fas, FasL or both, or whether they block Fas signal transduction. We have found that CsA- and FK506-treated cells did not exhibit transcription of FasL mRNA after activation and were lacking functional FasL protein on their surface as determined by staining and the ability to induce apoptosis in Fas$^+$ target cells. In contrast, no inhibition of the elevated Fas mRNA expression was observed in cells activated in the presence of CsA or FK506. Surprisingly, however, cell surface Fas levels were consistently lower on cells activated in the presence of immunosuppressive drugs than on activated cells, suggesting that Fas expression is regulated at several levels. Nevertheless, cells activated in the presence of CsA or FK506 underwent apoptosis upon treatment with anti-Fas antibody, while unactivated cells did not. Furthermore, CsA and FK506 do not interfere with Fas signaling since anti-Fas induced apoptosis in Fas$^+$ target cells was unaffected by these drugs. We therefore conclude that CsA and FK506 block activation-induced apoptosis in T cell hybridomas predominantly by interfering with activation signals leading to FasL expression and, further, that the regulation of the expression of Fas and FasL on activated T cells is differentially controlled.

Introduction

Activation-induced cell death, in which signals normally associated with lymphocyte stimulation instead result in the demise of the cell, has been proposed as a mechanism of deletion of antigen-specific lymphocytes. It has been demonstrated that activation-induced cell death occurs by the process of apoptosis with its characteristic features that include membrane blebbing, chromatin condensation, formation of apoptotic bodies, fragmentation of nuclear DNA and finally the engulfment of the apoptotic cell by phagocytes (reviewed in 1). Thymic T cells, especially immature cells at the CD4/CD8 double-positive stage, undergo apoptotic cell death when activated by TCR cross-linking *in vitro* (2,3) as well as *in vivo* (4,5). Peripheral T lymphocytes were thought to be resistant to activation-induced apoptosis since activation of the TCR usually leads to proliferation and clonal expansion; however, recent evidence shows that mature activated peripheral T lymphocytes may die by apoptosis upon religation of the TCR (peripheral deletion) (reviewed in 6). Interestingly, freshly isolated peripheral T cells are quite resistant to activation-induced cell death, but acquire the capability to undergo activation-induced apoptosis upon primary activation by antigen presented on antigen-presenting cells (7–9). It has been proposed that activation-induced apoptosis is at least one reason for the loss of CD4$^+$ T cells in pathology of AIDS

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(10–13). Activation-induced apoptosis, therefore, seems to be an important mechanism to control the development and expansion of immunocompetent T lymphocytes.

Murine T cell hybridomas are a well documented model system for the study of activation-induced apoptosis. Most T cell hybridomas die within hours after activation by presentation of specific antigen, mitogens, anti-TCR or anti-CD3 antibodies or the combination of phorbol ester and ionomycin (reviewed in 14). Recently, we (15) and others (16,17) have characterized this form of activation-induced apoptosis and have found that it involves *de novo* synthesis of two molecules: Fas (CD95) and its ligand (FasL). Fas is a type I transmembrane molecule of the tumor necrosis factor (TNF) receptor family of proteins with widespread expression among hematopoietic cells (18,19). Its engagement by anti-Fas antibodies (20,21) or the FasL (22) leads to apoptotic cell death of the target cell. Fas- and perforin/granzyme B-mediated apoptosis of target cells seems to account for the two major killing mechanisms of cytotoxic T cells (23–28). We have demonstrated that activation-induced co-expression of Fas and its ligand on T cell hybridomas leads to apoptotic cell death by functional interaction of ligand and receptor, which induces apoptosis in a cell autonomous manner, i.e. even on a single cell (15).

Cyclosporin A (CsA) and FK506 are immunosuppressive drugs that potently inhibit activation-induced apoptosis in murine T cell hybridomas (29–32). Since activation-induced apoptosis in T cell hybridomas is the result of interaction of *de novo* synthesized Fas and FasL, CsA and FK506 might block activation-induced apoptosis in these cells at any of three potential levels: they might interfere with the signal transduction events leading (i) from TCR ligation to Fas expression, (ii) from TCR ligation to FasL expression or (iii) from Fas ligation to the apoptotic process itself. Here we demonstrate that CsA and FK506 do not affect Fas-induced cell death, nor do they prevent functional Fas expression after cell activation. However, these drugs potently block activation-induced expression of FasL and thus block activation-induced cell death by inhibiting functional Fas/FasL interaction.

Methods

Reagents and cells

The T cell hybridoma line A1.1 has been described previously (33). Parental L1210 cells and L1210 expressing murine Fas (L1210.Fas) (25) were kindly provided by P. Golstein (INSERM-CNRS, Marseille, France). Jurkat E6 were obtained from ATCC (Rockville, MD). Cells were cultured in RPMI 1640, 5% FCS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM HEPES (complete medium). Phorbol myristate acetate (PMA) was purchased from Sigma (St Louis, MO) and ionomycin from Calbiochem (La Jolla, CA). Hamster anti-mouse CD3ε (145–2C11) was purified from culture supernatant by Protein A-affinity chromatography (34). Normal mouse IgG was purified from serum of 10- to 12-week-old mice by Protein G-affinity purification. Hamster anti-murine Fas (Jo2) was purchased from PharMingen (La Jolla, CA). Normal hamster IgG (NHIgG) was purified from serum (Sigma) by Protein G-affinity purification. Phycoerythrin-conjugated goat anti-hamster Ig was obtained from Accurate (Westbury, NY), unconjugated goat anti-hamster Ig from Fisher Biotech (Pittsburgh, PA), biotinylated rabbit anti-human IgG from Dako (Carpenteria, CA) and streptavidin–TriColor from Caltag (San Francisco, CA). Anti-human Fas (CH-11) was obtained from Kamiya (Thousand Oaks, CA) Generation and purification of the chimeric Fas–Fc proteins, consisting of the extracellular ligand-binding domain of human or mouse Fas antigen and the Fc portion of human IgG has been described (15). Murine Fas–Fc was kindly provided by D. Lynch (Immunex, Seattle, WA). CsA and FK506 were generously provided by P. Nelson (Boston, MA). Stock solutions of CsA were stored in ethanol; PMA, ionomycin and FK506 were dissolved in DMSO Final concentrations of ethanol or DMSO in assays were <0.1%.

Assessment of activation-induced cell death in T cell hybridomas

To assess the effect of CsA, FK506 or blocking Fas and FasL interaction by soluble Fas–Fc on activation-induced cell death, A1.1 T hybridoma cells were cultured in anti-CD3-coated 96-well plates (3 μg/ml) or with 10 ng/ml PMA and 150 ng/ml ionomycin (5×10^4 cells/well, 100 μl) in complete medium. Inhibitory drugs or Fas–Fc were added immediately in appropriate concentrations to the assay. Cell death was assessed by propidium iodide (PI) dye uptake (35) Accordingly, cells were harvested after 18 h and 10 μg/ml PI (Sigma) was added immediately before flow cytometrical analysis using a FACScan (Becton Dickinson, Mountain View, CA). Calibration of the cytometer was performed with fresh viable A1.1 cells by eye The percentage of PI-positive (dead) cells was calculated using Lysys II software. Cells were scored as PI-positive as indicated in Fig 3(d), using fresh viable A1.1 and cells cultured overnight in anti-CD3 coated plates to set gates. Assays were performed in triplicate and 5000 events were analyzed per condition.

Functional assay for FasL expression

Activation-induced FasL expression on A1.1 cells was assessed by determining the ability of these cells to cause DNA fragmentation in target cells. Parental L1210 cells, L1210.Fas cells, constitutively expressing murine Fas (25) or Jurkat were labeled at 10^6 cell/ml with 5 μCi/ml [^{3}H]thymidine for 2 h Unincorporated [^{3}H]thymidine was removed by two washes with HBSS. Target cells (2×10^4) were incubated with 4×10^4 A1.1 in 200 μl complete medium in flat-bottom 96-well plates, previously coated with 3 μg/ml anti-CD3 or untreated, in the presence or absence of CsA or FK506. Alternatively, cells were activated with 10 ng/ml PMA and 150 ng/ml ionomycin. No DNA fragmentation was observed when target cells were treated with anti-CD3 or PMA/ionomycin alone in the absence of A1.1. After 7–9 h, cells were harvested using a Skatron cell harvester and [^{3}H]thymidine-labeled unfragmented DNA was determined in a liquid scintillation counter. DNA fragmentation was calculated as follows: % DNA fragmentation = 100×(1 – c.p.m. experimental group/ c.p.m. control group) ± SD. Assays were performed in quadruplicate.

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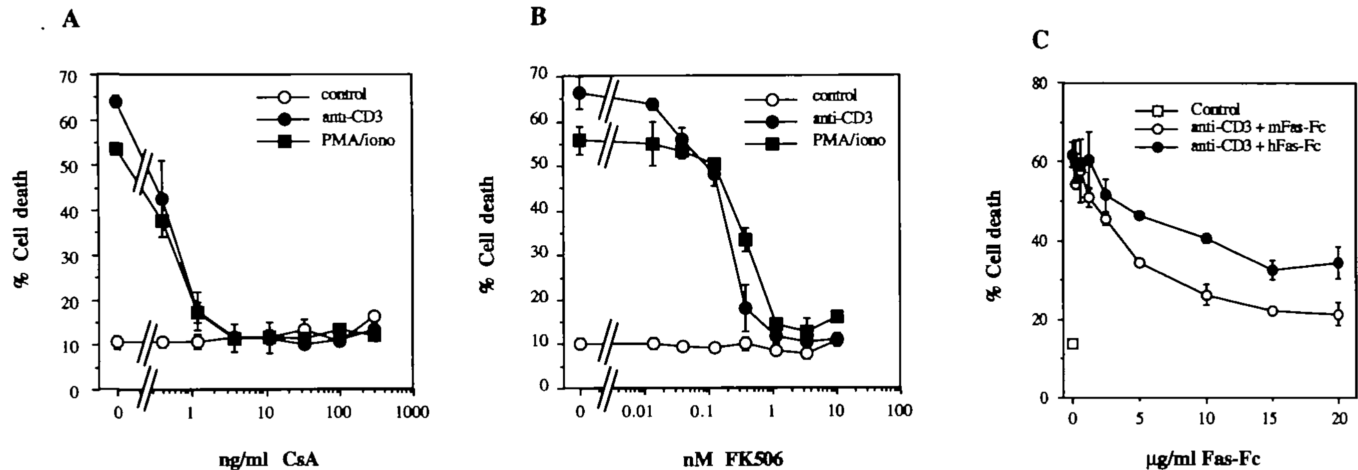

Fig. 1. Activation-induced apoptosis is blocked by CsA, FK506 and Fas–Fc A1 1 cells were cultured in control medium, in anti-CD3-coated 96-well plates or with PMA/ionomycin (5×10^4 cells/well), in the presence or absence of increasing concentrations of CsA (A), FK506 (B), or human or murine Fas–Fc (C) After 18 h cells were harvested and cell death as assessed by membrane integrity was measured as described in Methods (A and B) Open circles, control medium, closed circles, anti-CD3, closed squares, PMA/ionomycin (C) Open squares, control medium, open circles, anti-CD3 plus mFas–Fc, closed circles, anti-CD3 plus hFas–Fc Mean values of triplicates ± SD of representative experiments are shown

RNase protection assay (RPA) for Fas and FasL

RPA for Fas and FasL was performed as described (15) using a commercial RPA kit (Ambion, Austin, TX) according to the manufacturer's protocol Briefly, Fas and FasL cDNA (kindly provided by P. Golstein, INSERM, Marseille, France and S. Nagata, Osaka Bioscience Institute, Osaka, Japan respectively), and β-actin as a positive control, were cloned into pBluescript II (Invitrogen, La Jolla, CA). A1.1 (1×10^7 cells total per condition) were cultured for 4 h in either control medium or on anti-CD3-coated tissue plates, in the presence or absence of CsA (100 ng/ml) or FK506 (4 nM), and total RNA was isolated. Between 10 and 30 µg of RNA was hybridized with radiolabeled antisense RNA transcripts, prior to digestion with RNase T1. Samples were then separated by urea/SDS–PAGE and gels were exposed to X-ray film.

Surface staining of Fas and FasL

A1.1 were cultured in control medium, in anti-CD3-coated 24-well plates or in the presence of PMA (10 ng/ml) and ionomycin (150 ng/ml) at 5×10^5 cells/ml in complete medium, in the presence or absence of CsA (100 ng/ml) or FK506 (4 nM) After 4–5 h cells were harvested and briefly washed in PBS/1% calf serum/0.05% sodium azide (wash buffer). Non-specific antibody binding was blocked by incubating the cells with 50 µg/ml normal mouse IgG for 15 min at room temperature. Then, anti-mouse Fas Jo2 was added at 5 µg/ml and cells were incubated at 4°C for 30 min. After two washes with wash buffer, bound anti-Fas was detected by staining with 2.5 µg/ml phycoerythrin-conjugated goat anti-hamster secondary antibody (1:200) for 30 min at 4°C. After two washes, cells were fixed in PBS/1% formaldehyde prior to analysis on a FACScan flow cytometer.

For staining of cell-surface FasL, cells were cultured and blocked for non-specific antibody binding as described above. Then, 40 µg/ml hFas–Fc was added directly and cells were incubated for 30 min at 4°C After two washes, bound hFas–Fc was detected using a secondary biotinylated rabbit anti-human IgG (1:200) and streptavidin–TriColor fluorescent reagent (1:20), and cells were analyzed using a FACScan

Demonstration of functional Fas on T cell hybridomas

To determine whether A1.1 express functional Fas after blocking activation-induced cell death by drugs, cells were incubated at 5×10^4/well in an anti-CD3-coated 96-well plate for 4 h at 37°C, in the presence or absence of CsA (100 ng/ml) or FK506 (4 nM). Cells were then pelleted by centrifugation and supernatant was removed. Cells were resuspended in 50 µl complete medium and incubated in medium only, normal hamster IgG or anti-Fas Jo2 (2.5 µg/ml) for 20 min at 4° C. After two washes, medium was replenished with appropriate drugs. Cells were then further incubated at 37°C overnight and cell death was assessed by permeability for PI as described above. Assays were performed in triplicate and cells were pooled prior to analysis of cell death; 5000 events were analyzed per condition.

Assessment of anti-Fas-induced cell death in Jurkat and L1210.Fas cells

To test whether CsA and FK506 interfere with Fas-induced cell death, Jurkat and L1210.Fas cells were cultured at 5×10^5 cells/ml in a flat-bottom 96-well plate in control medium, with anti-human Fas antibody (Jurkat) (CH-11, 100 ng/ml) or with anti-murine Fas (L1210.Fas) (Jo2, 2.5 µg/ml). In the case of L1210.Fas, plates were precoated with 5 µg/ml goat anti-hamster. CsA (200 ng/ml) or FK506 (10 nM) were added and 12 h later cells were harvested and were analyzed for membrane integrity by PI uptake.

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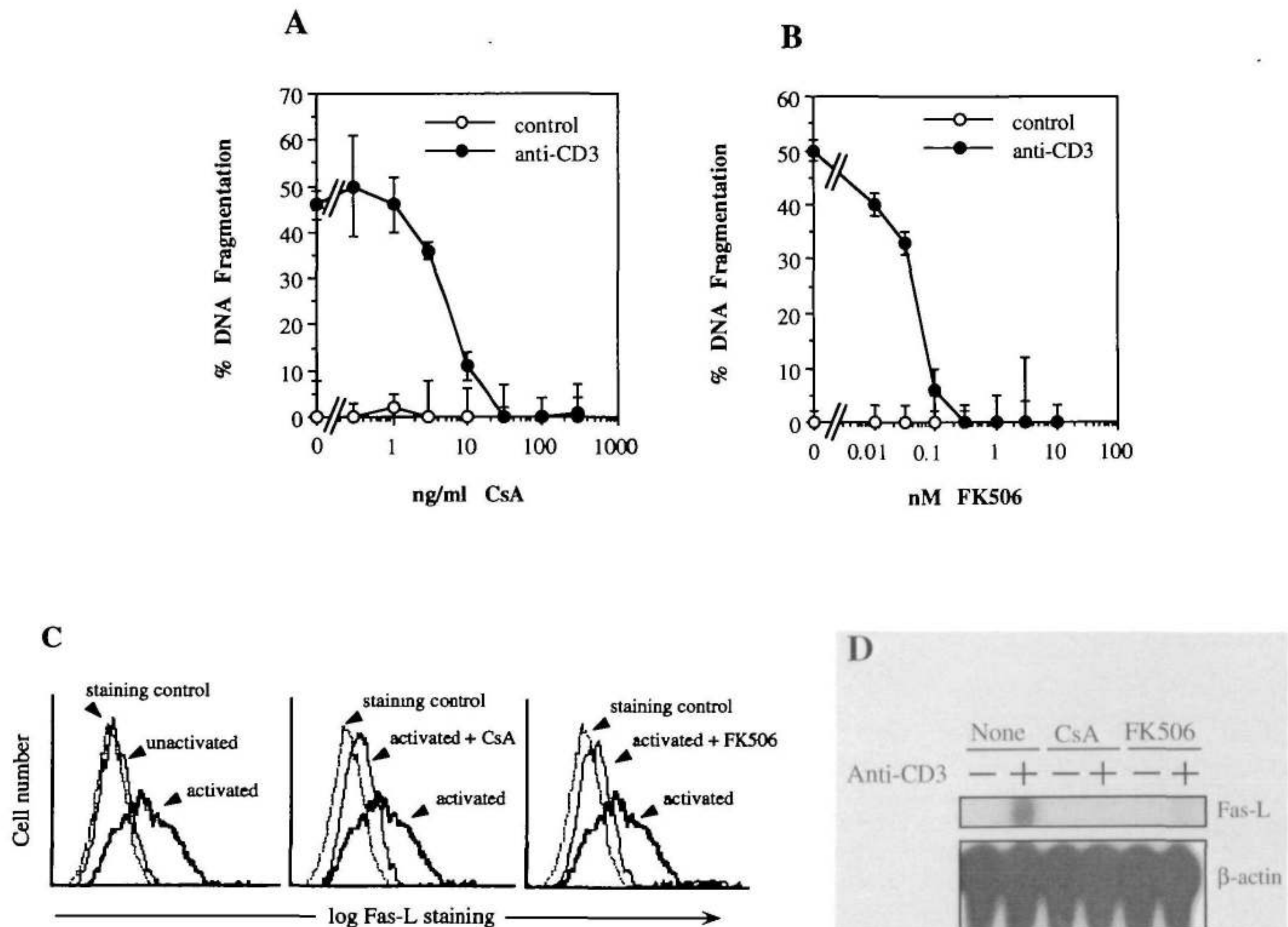

Fig. 2. CsA and FK506 block activation-induced FasL expression A1 1 cells were co-cultured with [^{3}H]thymidine-labeled L1210.Fas cells in control medium or in anti-CD3-coated 96-well plates CsA (A) or FK506 (B) was added at the indicated concentrations After 9 h DNA fragmentation in L1210 Fas target cells was assessed as described in methods. Open circles, control medium; closed circles, anti-CD3 Mean values of quadruplicates ± SD are shown (C) Staining of cell surface Fas ligand A1 1 cells were cultured in control medium or activated with 10 ng/ml PMA and 150 ng/ml ionomycin, in the presence or absence of CsA (100 ng/ml) or FK506 (4 nM). After 5 h, cells were stained with hFas–Fc and analyzed by flow cytometry Dotted lines show staining control (D) FasL mRNA expression A1 1 cells were cultured in control medium or on anti-CD3-coated plates for 4 h, in the presence or absence of CsA (100 ng/ml) or FK506 (4 nM), and total RNA was prepared RPA was performed as described in Methods. The upper panel shows FasL mRNA, the lower panel shows β-actin control.

Results

Immunosuppressive drugs and soluble Fas–Fc block activation-induced cell death in T hybridomas

In order to investigate a possible role for CsA and FK506 in regulating Fas and/or FasL expression, the murine T cell hybridoma A1.1 was stimulated with either immobilized anti-CD3 or a combination of PMA and ionomycin. As demonstrated in Fig. 1, extensive cell death was observed 18 h after stimulation with either agent. Approximately 80% of the cells became permeable for PI after either anti-CD3 stimulation or activation with PMA/ionomycin. Both CsA and FK506 were able to block activation-induced cell death in a dose-dependent manner. CsA protected from cell death at an ED_{50} of ~2 ng/ml. Similarly, FK506 inhibited cell death at an ED_{50} 0.3 nM. Effective concentrations of CsA and FK506 were similar to those previously reported to block IL-2 synthesis in peripheral T cells (36). Both drugs showed little or no toxicity when added alone at the concentrations tested. Only a slight increase of cell death over background was observed with 300 ng/ml CsA. Thus, these results confirm previous findings that the immunosuppressive drugs CsA and FK506 block activation-induced cell death (29–32).

We have recently found that activation of T cell hybridomas leads to expression of Fas and FasL (15). At least in the case of A1.1, both molecules are apparently absent from unactivated cells, but are rapidly expressed after TCR ligation or treatment with PMA/ionomycin. Furthermore, activation-induced apoptosis in T cell hybridomas was inhibited when a soluble chimeric protein, consisting of the extracellular ligand-binding domain of the Fas molecule and the Fc portion of human IgG (Fas–Fc), was added to the culture (15). Since we used human Fas–Fc for these studies, we reconfirmed our previous findings by comparing the effect of human Fas–

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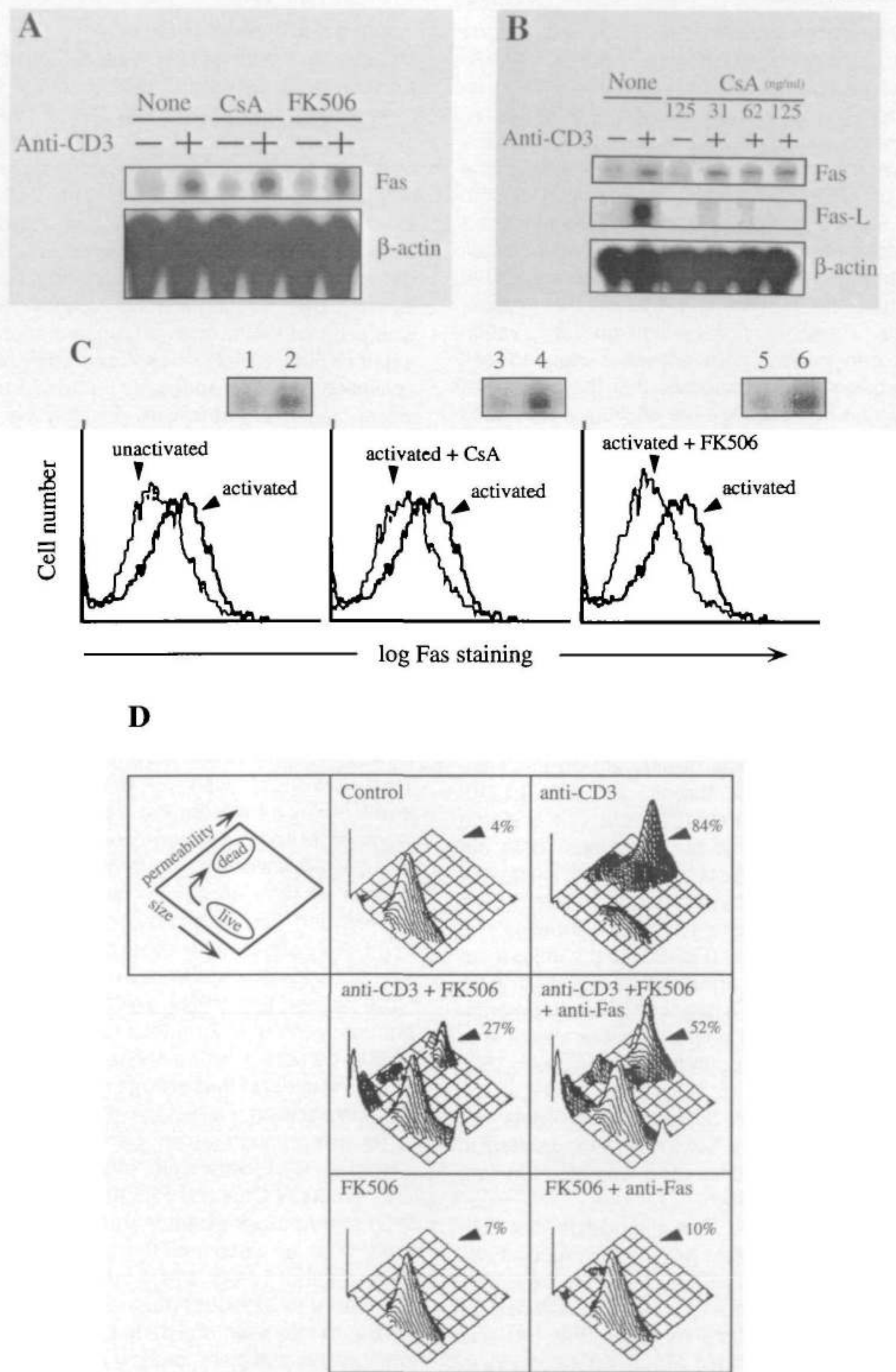

Fig. 3. Effect of CsA and FK506 on activation-induced Fas expression. (A) A1.1 cells were cultured for 4 h in control medium or on anti-CD3-coated plates, in the presence or absence of CsA (100 ng/ml) or FK506 (4 nM). Fas mRNA (upper panel) was analyzed by RPA and compared to β-actin mRNA (lower panel). (B) A1.1 cells were incubated on anti-CD3-coated plates or control medium with increasing concentrations of CsA. Fas (upper panel), FasL (middle panel) and β-actin (lower panel) mRNA levels were analyzed by RPA (C) Staining for cell surface Fas A1.1 cells were activated with anti-CD3 in the presence or absence of CsA or FK506 as described above for 4 h, stained with anti-Fas and then analyzed by flow cytometry. Dotted lines show staining control. Insets show Fas mRNA levels of the same experiment. (1) unstimulated; (2) anti-CD3, (3) CsA; (4) anti-CD3 plus CsA; (5) FK506; (6) anti-CD3 plus FK506. (D) Induction of apoptosis in A1.1 by anti-Fas A1.1 cells were cultured in control medium or on anti-CD3 coated plates, in the presence or absence of 4 nM FK506 After 4 h, cells were treated with anti-Fas or control medium for 30 min Cells were then cultured for an additional 14 h in the presence or absence of FK506 (4 nM) and cell death was analyzed. Cells were scored as live or dead by their permeability for PI, as indicated in the upper left corner of the figure. Percentages of PI-positive cells (arrows) are indicated. Experimental triplicates were pooled and 5000 events were analyzed. A typical experiment is shown

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Fc (hFas–Fc) on activation-induced cell death with murine Fas–Fc (mFas–Fc). Therefore, A1.1 were cultured on anti-CD3-coated plates, or alternatively activated with PMA/ionomycin, in the presence or absence of Fas–Fc (2.5–30 µg/ml). After 18 h, cell death was assessed as loss of membrane integrity. As shown in Fig. 1(C), activation by anti-CD3 alone led to the death of ~55% of the cells. The addition of both forms of soluble Fas–Fc protected from activation-induced cell death in a dose-dependent manner and reduced cell death to near background levels. The mFas–Fc was somewhat more effective than the human in preventing activation-induced apoptosis. These findings confirm our observation that soluble Fas can protect from activation-induced cell death in T cell hybridomas and suggest that the binding of murine FasL to murine Fas occurs more efficiently than does its binding to human Fas.

CsA and FK506 prevent activation-induced FasL expression

Since activation-induced cell death in murine T cell hybridomas appears to be dependent on Fas and FasL expression (Fig. 1C) (15), we asked whether CsA and FK506 mediate their inhibitory effect by blocking expression of Fas and/or FasL. FasL expression was measured functionally using a simple cellular assay of Fas-induced DNA fragmentation in Fas$^+$ target cells (15). When [^{3}H]thymidine-labeled L1210.Fas cells were co-cultured with activated A1.1 cells, significant DNA fragmentation was induced in these cells (Fig. 2A and B). This process was dependent on Fas/FasL interaction, since the parental target cell line L1210, expressing only low levels of Fas (25), did not undergo apoptotic cell death under the same conditions (data not shown). Only basal DNA fragmentation in target cells was observed when A1.1 were not activated or when target cells were incubated with stimuli alone (data not shown). The addition of CsA (Fig. 2A) or FK506 (Fig. 2B) to the culture not only blocked activation-induced apoptotic cell death of A1.1 cells, but also efficiently prevented target cell DNA fragmentation. The effective dose range of both drugs was identical to that observed to inhibit activation-induced cell death (Fig. 1A and B). Incubation with drugs alone had no effect on background DNA fragmentation of target cells (data not shown). Activation of A1.1 with PMA/ionomycin resulted in similar target cell DNA fragmentation and was also blocked by CsA and FK506 (data not shown).

The inhibition of Fas-mediated killing suggests that CsA and FK506 may negatively regulate activation-induced FasL expression in murine T cell hybridomas. To address this question more directly, activated A1.1 cells were stained for FasL expression using the soluble receptor (Fas–Fc) and appropriate secondary reagents (15). Figure 2(C) shows that PMA/ionomycin-activated A1.1 express FasL as revealed by binding of soluble hFas–Fc to these cells In contrast, no Fas–Fc binding was observed on unactivated cells as compared with background staining with secondary reagents only, indicating that unactivated A1.1 do not express surface FasL molecules. The addition of CsA or FK506 to PMA/ionomycin-activated A1.1, at concentrations that entirely blocked Fas-mediated killing of target cells (Fig. 2A and B), efficiently reduced the binding of soluble Fas–Fc to the cell surface, thus confirming that CsA and FK506 do indeed block activation-induced FasL expression in A1.1 (Fig. 2C). We similarly observed induction of FasL by anti-CD3 and inhibition of FasL expression by CsA and FK506 (data not shown).

As shown in Fig. 2(D), CsA and FK506 affect activation-induced FasL expression by interfering with elevation of FasL mRNA We employed the RPA for detection of FasL mRNA in activated A1.1 after treatment with CsA and FK506. A1.1 express FasL mRNA after 4 h post-activation with anti-CD3, whereas no mRNA was detectable in unstimulated cells. As demonstrated by RPA, the strong induction of FasL message by anti-CD3 activation was significantly decreased when cells where treated with optimal concentrations of CsA (200 ng/ml) or FK506 (10 nM). Thus, CsA and FK506 appear to inhibit activation-induced apoptosis in this T cell hybridoma by influencing levels of mRNA, possibly via inhibition of induction of FasL transcription.

Activation-induced Fas expression is only partially affected by CsA and FK506

The question remained as to whether CsA and FK506 not only interfere with activation-induced FasL expression but also block expression of Fas in A1.1, since both FasL as well as Fas expression are induced after anti-CD3 activation (15). The effect of CsA and FK506 on activation-induced Fas mRNA was therefore analyzed by RPA As shown in Fig. 3(A), unactivated cells expressed low levels of Fas mRNA which was significantly increased after cell activation with anti-CD3. Treatment of cells with CsA or FK506 had no effect on basal levels of Fas mRNA nor did it prevent the activation-induced increase. In another experiment (Fig. 3B), cells were activated by anti-CD3 antibodies in the presence of a range of concentrations of CsA and RNA from these cells was analyzed for Fas and FasL expression. Figure 3(B) shows that CsA completely abolished activation-induced FasL mRNA expression at concentrations from 31 to 125 ng/ml. In contrast, anti-CD3-induced Fas mRNA expression was not affected at any concentration of CsA. It thus seems very likely that CsA and FK506 do not inhibit functional Fas expression and that the inhibitory effect of these drugs on cell death is due to blocking FasL expression.

To detect changes in cell surface expression of Fas, activated A1.1 cells were stained with anti-Fas antibodies and effects of CsA and FK506 were again examined. Figure 3(C) shows a significant increase in Fas expression in A1.1 cells after activation of the cells with anti-CD3. Although expression of Fas on this T cell hybridoma was quite low as compared to activated murine spleen cells (not shown), it is similar to that seen on L1210.Fas cells (not shown) and thus presumably sufficient to allow Fas-mediated cell death upon FasL expression Surprisingly, A1.1 activation in the presence of CsA or FK506 resulted in partial inhibition of cell surface Fas expression. This partial inhibition was consistently observed in a number of experiments. Since this inhibition by CsA and FK506 of activation-induced cell surface Fas expression contrasts with the lack of inhibition at the mRNA level, we examined the effect of CsA and FK506 on activation-induced Fas expression by RPA and antibody staining in parallel. As shown in Fig. 3(C), CsA and FK506 significantly reduced activation-induced cell surface Fas expression. However, no

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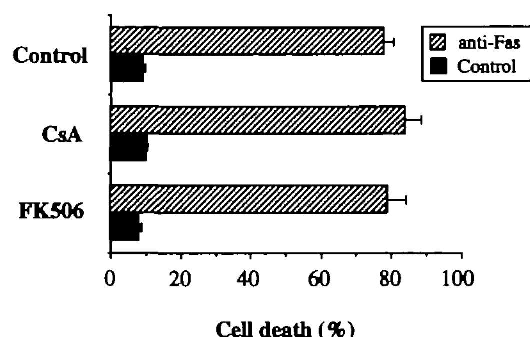

Fig. 4. CsA and FK506 do not affect Fas-mediated apoptosis Jurkat cells were cultured in control medium, with CsA (200 ng/ml) or with FK506 (10 nM) Apoptosis was induced by adding 100 ng/ml of anti-Fas After 12 h, cells were analyzed for PI permeability Mean values of triplicates ± SD of a typical experiment are shown

inhibition of Fas mRNA expression was observed when the same cells were analyzed by RPA (Fig. 3C, inset)

The discrepancy between the effects of CsA and FK506 on Fas mRNA synthesis and surface protein expression led us to ask whether A1.1 express functional Fas on their surface after activation in the presence of CsA or FK506. Cells were therefore activated with anti-CD3 in the presence or absence of CsA or FK506. After 4 h, where Fas cell surface expression is observed (Fig. 3C) (15), anti-murine Fas antibody was added and cell death was assessed 14 h later. As shown in Fig. 3(D), activation of A1.1 resulted in extensive cell death, which was blocked by addition of FK506. However, if anti-Fas was added to these protected cells, a significant increase in cell death was observed. Control hamster antibodies (NHIgG) had no effect on increase of cell death (data not shown). No apoptosis was observed in unactivated A1.1 ± FK506, when treated with anti-Fas. Similar levels of cell death were achieved when CsA-blocked cell death was overcome by addition of anti-Fas (data not shown). These findings indicate that CsA and FK506 do not completely abrogate activation-induced Fas expression, since anti-Fas-induced apoptosis can still occur. Further, since anti-Fas treatment induced apoptosis in these cells, it suggests that these drugs do not block Fas-induced apoptosis. This was examined in more detail below.

CsA and FK506 do not interfere with anti-Fas-induced cell death

The results from the experiments described above suggested that CsA and FK506 affect activation-induced apoptosis by preventing FasL expression, rather than by blocking Fas expression or Fas signaling leading to apoptosis. However, at least partially inhibitory effects of these drugs on Fas-induced apoptosis could not be excluded, since anti-Fas did not fully compensate for the drug-induced block in cell death in activated A1.1 (Fig. 3D). Jurkat cells constitutively express high levels of Fas molecules on their surface and readily die by apoptosis upon cross-linking with anti-Fas. A relatively high percentage of apoptosis was observed when Jurkat cells were treated with anti-Fas (77%), as compared with spontaneous death in culture (9%). The addition of CsA or FK506 did not affect in any way the amount of anti-Fas-induced cell death in these cells (84 and 79% respectively) nor did the drugs alone increase basal death of control cells (Fig. 4). In contrast, activated A1.1 were found to induce DNA fragmentation in Jurkat cells, a process which was blocked by the addition of CsA and FK506, most likely via inhibition of FasL expression in A1.1 (data not shown). Similar findings were made with L1210 constitutively expressing murine Fas (L1210.Fas) significant cell death was observed by addition of anti-Fas, as compared with goat anti-hamster only or NHIgG as control. The addition of either CsA or FK506 had no significant effect on anti-Fas induced apoptosis (data not shown). Thus, CsA and FK506 do not block anti-Fas-induced apoptosis in either human or murine cells.

Discussion

Murine T hybridoma cells are a widely used system for the study of the mechanisms of activation-induced apoptosis and a model for peripheral deletion. We and other groups have previously shown that activation-induced apoptosis in murine T cell hybridomas is blocked by CsA and FK506 (29–32). Recently, we (15) and others (16) dissected the mechanism of activation-induced cell death in different T cell hybridomas, and found that this form of cell death proceeds via interaction of activation-induced Fas and its ligand FasL. Fas and FasL are virtually absent on unactivated cells, but are rapidly induced after activation. Since this process requires *de novo* protein synthesis, several agents might regulate Fas or FasL expression by interfering in the signal transduction pathway leading to gene transcription. The goal of this study was therefore to determine whether immunosuppressive drugs, such as CsA and FK506, block activation-induced apoptosis by interfering with Fas-dependent cell death. Our data strongly indicate that CsA and FK506 block anti-CD3 or PMA/ionomycin-induced expression of FasL in the murine T cell hybridoma A1.1. This was observed for FasL mRNA as well as cell surface expression. Our data exclude the possibility that CsA and FK506 might interfere with the Fas signaling pathway since anti-Fas-induced cell death in L1210.Fas and Jurkat cells was unaffected by treatment with these drugs, at concentrations that completely blocked activation-induced apoptosis in T cell hybridomas or FasL-mediated DNA fragmentation in target cells. In addition, CsA and FK506 blocked activation-induced death in A1.1 cells, but could not prevent anti-Fas-induced apoptosis (Fig. 3D). We conclude from these data that CsA and FK506 inhibit activation-induced cell death in murine T cell hybridomas predominantly by blocking FasL expression and thus preventing Fas/FasL interaction, leading to activation of the apoptosis program.

Upon activation of T cell hybridomas, not only is FasL expression strongly induced, but also expression of its receptor, Fas. However, we observed contrasting effects of CsA and FK506 on activation-induced expression of Fas protein versus mRNA. Fas mRNA expression was completely unaffected by treatment of the cells with these drugs, as demonstrated by RPA (Fig. 3A–C). Surprisingly, we repeatedly observed at least partial inhibition of activation-induced Fas cell surface expression (Fig. 3C), although the remaining

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activation-induced Fas was apparently functional since significant cell death was observed after cross-linking cell surface Fas molecules (Fig. 3D).

The levels of Fas expressed on unactivated A1.1 cells and those that were activated in the presence of CsA or FK506 were approximately equal (Fig. 3C), yet only the latter were susceptible to cell death induced by anti-Fas antibody (Fig. 3D). This raises the interesting possibility that activation of the hybridomas increased the susceptibility to Fas-induced death in a manner which is resistant to CsA or FK506. This might be through the inhibition of molecules which block Fas-induced death, such as FAP-1 (37). In this regard, it is interesting that activation of some T lines leads to down-regulation of FAP-1 expression (T. Brunner, unpublished observations); however, we have been unable to detect FAP-1 in A1.1. Alternatively, activation might up-regulate elements of the Fas-induced signal transduction pathway which lead to apoptosis or increase susceptibility via other interacting elements. Thus, while activation of resting T cells may induce a resistance to Fas-induced apoptosis (38), it may also induce susceptibility under other circumstances.

The observation that activation-induced Fas mRNA expression is unaffected by CsA and FK506 while significant inhibition of Fas cell surface levels was detected is intriguing and rather difficult to understand. The CsA-binding protein cyclophilin has been shown to mediate certain protein folding processes and to exhibit chaperone activity, a process which is sensitive to CsA (reviewed in 39) However, ~100-fold higher concentrations of CsA are required to block these processes, as compared with inhibition of activation-induced cell death or IL-2 synthesis. It is also possible that activation-induced Fas protein expression is unaffected by CsA and FK506. However, the transport of *de novo* synthesized Fas to the cell surface might be facilitated by (an) other molecule(s) whose expression, possibly induced by the cell activation process, might be CsA and FK506 sensitive. A third possibility is that CsA and FK506 induce proteolytic shedding of activation-induced surface expressed Fas by metalloproteases. An ability of activation to induce a metalloprotease responsible for shedding of the 80 kDa TNF receptor has been described in T cells (40), although its sensitivity to (or enhancement by?) CsA or FK506 is not known. Further analysis as to whether *de novo* synthesized Fas is intracellularly retained, shed or even degraded after treatment of the cells with CsA or FK506 might help to resolve this unanswered question.

A proposed target molecule for the action of CsA and FK506 is the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin (41). Calcineurin has been demonstrated to promote translocation of transcription factors, such as Nur77, NF-κB and NFAT, to the nucleus. In addition, it has been shown that calcineurin activity correlates with anti-CD3-induced apoptosis in murine T cell hybridomas and with antigen-mediated cytotoxic responses of murine T cell clones (32,42). Nur77, an orphan transcription factor of the steroid/thyroid superfamily, has been demonstrated to be critically involved in activation-induced apoptosis of murine T cell hybridomas (43,44). Recently, it has been shown that CsA interferes with the DNA binding activity of Nur77 and might therefore block activation-induced apoptosis in these cells (45). So far, no target gene for Nur77 activity has been identified. It will therefore be interesting to determine whether FasL expression is regulated by Nur77.

Although not directly proven, our data suggest that calcineurin might be somehow involved in TCR signaling leading to FasL expression and therefore to activation-induced cell death. Activation of calcineurin by elevated intracellular Ca^{2+} and calmodulin also leads to dephosphorylation of the transcription factor NFAT (46,47). However, no NFAT binding sequence has been identified in 450 bases upstream of the FasL promoter (48). Thus, either there is an NFAT binding site further upstream in the FasL promoter or other calcineurin-dependent transcription factors might be responsible for FasL transcription. Calcineurin not only controls NFAT activation, but also regulates another transcription factor, NF-κB, together with a protein kinase C (PKC)-mediated signal (49). Since TCR activation as well as the combination of ionomycin and PMA induces a rise in intracellular calcium as well as activation of PKC, NF-κB may be one of the transcription factors regulating FasL expression. Indeed, a consensus NF-κB binding site has been identified in the FasL promoter (48).

Anel *et al.* (50) have recently reported that CsA and Ca^{2+}-free conditions negatively affect activation-induced FasL expression in murine $CD8^+$ cytotoxic T lymphocytes. Similarly, Dhein *et al.* (17) have found that activation of Jurkat cells leads to expression of FasL, a process which is CsA sensitive. Our observation that activation-induced cell death in murine $CD4^+$ T cell hybridomas is blocked by CsA and FK506 by preventing FasL expression is in agreement with these findings We observed, however, that neither PMA nor ionomycin alone induced expression of FasL in the T cell hybridoma A1.1 (unpublished results). In certain murine cytotoxic T lymphocyte clones, however, PMA alone seems to be sufficient to induce substantial expression of FasL, which is synergistically enhanced by ionomycin (50) The ability of PMA alone to induce FasL expression inversely correlated with the reduced ability of CsA to block PMA/ionomycin-induced FasL expression in these clones. Thus, CsA only partially blocked activation-induced FasL expression in the PMA-responsive $CD8^+$ T cell clones. However, further dissection of activation signal transduction pathways will be necessary to fully elucidate the role of calcineurin and PKC-derived signals in FasL gene expression.

Since activation-induced Fas mRNA expression was CsA and FK506 insensitive in our studies, it seems that the signal transduction pathway leading to Fas expression is different from that of its ligand. Stimuli leading only to expression of one or the other would thus be extremely helpful to dissect the relevant signal transduction pathways. Since Fas is widely expressed in hematopoietic and non-hematopoietic cells (20,21,51), and also in non-activated cells such as polymorphonuclear granulocytes (52), it seems that the expression of Fas is not so tightly regulated by activation signals as compared with its ligand. Thus, regulation of activation-induced apoptosis, executed by interaction of Fas and FasL as described in this study, seems to be mainly regulated at the level of FasL expression.

Activation-induced apoptosis in murine T cell hybridomas is likely to be a model of peripheral deletion of activated T cells *in vivo*, where a role for Fas/FasL interactions has been suggested. This is based on studies employing mice with the

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lpr defect, which lack functional Fas expression due to insertion of a retroviral transposon into the *fas* gene (53). For example, antigen-induced peripheral deletion of $CD4^+$ T cells does not occur in TCR-transgenic *lpr* mice (54). Similarly, young *lpr* mice do not show peripheral deletion following administration of the superantigen, staphylococcal enterotoxin B (SEB) (55). Thus, activation-induced apoptosis responsible for peripheral deletion appears to be dependent upon Fas. Interestingly, treatment of SEB-injected mice with CsA had no effect on peripheral deletion (56), and this is consistent with our observation (Fig. 3) that activated, CsA-treated T hybridoma cells expressed Fas and were highly sensitive to Fas-induced apoptosis. However, the failure of CsA to inhibit peripheral deletion *in vivo* raises the tantalizing possibility that the FasL responsible for triggering this apoptosis *in vivo* is expressed in a CsA-insensitive manner, perhaps in an manner analogous to the CsA-resistant expression of FasL in some cytotoxic T lymphocyte clones, as discussed above (50).

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Abbreviations

CsA	cyclosporin A
Fas–Fc	Fas–Ig fusion protein
FasL	Fas ligand
PI	propidium iodide
PKC	protein kinase C
PMA	phorbol myristate acetate
RPA	RNase protection assay
SEB	staphylococcal enterotoxin B

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