

Comparison of Fas(Apo-1/CD95)- and perforin-mediated cytotoxicity in primary T lymphocytes

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Abstract

Cytolytic T lymphocytes kill target cells by two independent cytolytic mechanisms. One pathway depends on the polarized secretion of granule-stored proteins including perforin and granzymes, causing target cell death through membrane and DNA damage. The second cytolytic effector system relies on the interaction of the Fas ligand (FasL) on the effector cell with its receptor (Fas) on the target cell, leading to apoptotic cell death. Using mixed lymphocyte culture (MLC)-derived primary T lymphocytes of perforin-knockout and *gld* (with non-functional FasL) mice, the molecular basis of the two killing mechanisms was compared. The activity of both pathways was dependent on extracellular Ca^{2+} . Incubation of MLC-stimulated primary T cells with protein synthesis inhibitors prior to TCR triggering impaired FasL cell surface expression and abolished cytolytic activity, although the cells exhibited an intracellular pool of FasL. The perforin-dependent mechanism induced cell death more rapidly, although both pathways ultimately showed similar killing efficiencies. Both pathways induced comparable levels of DNA degradation, but Fas-induced membrane damage was less pronounced. We conclude that upon TCR triggering FasL may be recruited in part from pre-existing intracellular stores. However, efficient induction of target cell death still depends on the continuous biosynthesis of FasL molecules.

Introduction

Cytotoxic T lymphocytes (CTL) play a crucial role in the clearance of viral infections, tumor protection and graft rejection. Recently, the use of knockout mice has demonstrated the presence of two independent cytotoxic pathways in CTL (1,2). One mechanism consists of the directional release of cytotoxic granule-stored effector molecules upon specific interaction with a target cell, eventually leading to cell lysis and apoptosis (3). Perforin, a granule protein capable of forming transmembrane pores in a Ca^{2+} -dependent manner, is responsible for membrane damage (4,5), but fails to induce nuclear damage (6). Apoptosis is brought about by granzyme A or B, two granule proteins which belong to the family of serine proteases (7–10).

A second cytotoxic mechanism used by CTL depends on the interaction of the Fas ligand (FasL) and its cognate receptor (11). Fas-based killing can function in the absence of extracellular Ca^{2+} (11) and represents the major killing pathway of $CD4^+$ CTL (12,13). FasL is a 40 kDa class II

transmembrane protein belonging to the tumor necrosis factor family, and is expressed in a variety of different tissues particularly spleen, thymus, testis, kidney and lung (14). In addition to its role in CTL-mediated cytotoxicity, Fas is implicated in immune homeostasis as evidenced by the analysis of mutant mouse strains for FasL (*gld*) and Fas (*lpr*) (15). With age, these mice accumulate a large amount of non-malignant $CD4^+CD8^-$ T cells in their spleen and lymph nodes, and develop autoimmune disease pathology similar to systemic lupus erythematosus. Various studies examining the steps of T cell development regulated by the Fas/Apo-1 system have suggested that Fas is involved in the clonal deletion of lymphocytes in the periphery (16,17).

In the present study, we compared the mechanisms of perforin- and Fas-mediated killing. In our experimental system, Fas- and perforin-dependent pathways induced rapid cell death via apoptosis. Although FasL could be detected inside the CTL before TCR engagement, killing was dependent on

de novo protein synthesis, suggesting either the need for continuous ligand synthesis or the production of a putative FasL-activating protein

Methods

Cell lines and mice

A murine B lymphoma cell line, A20 (H-2^d, ATCC TIB 208), a murine fibrosarcoma clone, WEHI-164 clone 13 (H-2^d, ATCC CRL 1751), and an embryonic fibroblast cell line, 3T3.A31 (H-2^d, ATCC CRL 6588), were maintained in DMEM (Gibco, Basel, Switzerland) supplemented with 5% FCS, 10 mM HEPES (pH 7.4) and 5×10^{-5} M β -mercaptoethanol. To generate concanavalin A (Con A) blasts, spleen cells from DBA/2 mice (H-2^d) were freed from red blood cells and activated in complete DMEM supplemented with Con A (4 μ g/ml, Sigma) for 3 days. Alloreactive CTL were generated in a 5 day mixed lymphocyte culture (MLC) as previously described (18). Responder spleen cells (2.5×10^6 /ml) were obtained from adult (5- to 6-week-old) homozygous perforin-deficient mice (19) and C57/B6 *gld* mice (H-2^b; Jackson Laboratory, Bar Harbor, ME). Stimulator cells were irradiated spleen cells (2.5×10^6 /ml, 3000 rad; 1 rad = 0.01 Gy) from DBA/2 mice (Iffredo, Grenoble, France). All cells were cultured in a humid atmosphere containing 5% CO₂ at 3°C.

Cytotoxicity assay for MLC

Cytotoxicity assays were performed as previously described (20). To measure membrane damage, A20 cells or Con A blasts (10^6 cells in DMEM with 5% FCS) were labeled with 100 μ Ci [⁵¹Cr]sodium chromate (Amersham, Zürich, Switzerland) for 1 h in a final volume of 200 μ l. For DNA degradation, target cells (2×10^6) were labeled overnight with 2.5–5 μ Ci [³H]thymidine (Amersham). 3T3.A31 fibroblasts and WEHI-164 fibrosarcoma cells were labeled with [⁵¹Cr]sodium chromate (2 μ Ci/well) in 96-well plates for 12–16 h (21). Cytotoxicity was measured in a 7 h assay. For the inhibitor studies, MLC were preincubated with the various metabolic inhibitors (actinomycin D 2.5 μ g/ml; cycloheximide 2.5 μ g/ml; emetine 5 μ g/ml) for 45 min and either washed or directly mixed with labeled target cells. All inhibitors were obtained from Sigma (Buchs, Switzerland). In order to preactivate CTL, cells were incubated for 3 h in a mixture of phorbol 12-myristate 13-acetate (PMA; Sigma; final concentration of 5 ng/ml) and ionomycin (Sigma; 0.5 μ g/ml final).

Flow cytometric analysis

Primary MLC were taken at day 6 of stimulation and were centrifuged for 20 min at 2000 r.p.m. over a Ficoll-Hypaque cushion (Pharmacia, Location?) to eliminate dead cells. Washed buffy coat cells were stained with affinity-purified polyclonal antibodies against the FasL (PE62; M. Hahne *et al*, submitted for publication) followed by a fluorescein-coupled donkey anti-rabbit IgG antibody (Dianova, Hamburg, Germany). The samples were analyzed on a FACScan cytometer (Becton Dickinson, Mountain View, CA).

Immunofluorescence microscopy and Western blot analysis

Primary MLC at day 6 of stimulation were enriched for T cells by depletion of B220⁺ cells on a FACS cell sorter. Remaining

cells were cultured for 4 h in either the presence or absence of PMA (0.5 ng/ml) and ionomycin (0.5 μ g/ml). Subsequently, cells were washed with PBS and plated onto glass slides (pretreated with polylysine at 50 μ g/ml for 30 min). Cells were fixed and permeabilized in methanol at –20°C for 5 min. Slides were then incubated with the polyclonal antibody anti-PE62 (MAP-RGQSCNNQPLNHHKVVYMRNSKYPEDL), affinity-purified against the FasL peptide PE82 (GQSCNNQPLNHHKVVYMRNSKYPEDK). As a specificity control, the specific peptide PE82 (100 μ g/ml) or an unrelated peptide PE58 (EDEMPTLYVGNLS) were added during the first antibody incubation. Bound antibody was visualized with a donkey anti-rabbit FITC-conjugated secondary antibody (Dianova). Confocal microscopy was performed using the Zeiss LSM confocal laser system. Specimens were observed under epifluorescence illumination with a 488 nm scanning argon beam. For Western blotting, 2×10^6 sorted T lymphocytes were lysed in 50 μ l of cell lysis buffer (0.5% Triton X-100, 300 mM NaCl in 50 mM Tris-HCl, pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride. The postnuclear fraction was separated on a 10% SDS-PAGE and transferred to nitrocellulose. FasL was detected with the polyclonal antibody anti-PE62 (10 μ g/ml) and secondary anti-rabbit IgG antibody coupled to horseradish peroxidase (Sigma). Specificity controls were performed by adding either the specific peptide (PE82, 100 μ g/ml) or an unrelated peptide (PE58, 100 μ g/ml) during the first antibody incubation. Blots were developed using the Enhanced Chemiluminescence kit from Amersham.

Results

Fas-mediated cytotoxicity is dependent on extracellular calcium

Perforin-mediated cytotoxicity is strictly dependent on extracellular calcium, which is required for TCR-regulated granule exocytosis as well as the lytic activity of perforin itself (22). In contrast, Fas-based cytotoxicity of T cell clones has been reported to work in the absence of Ca²⁺ (1,10,17,23). In primary T cells, however, depletion of extracellular calcium with Mg²⁺-EGTA (3 mM/5 mM) abolished perforin- as well as Fas-mediated killing (Fig. 1). Fas-mediated cytotoxicity in primary MLC is specific and dependent on TCR engagement (1). To investigate if inhibition of TCR signaling, a calcium-dependent event, was responsible for the abrogation of Fas-mediated cytotoxicity, we preincubated effector cells for 3 h with PMA plus ionomycin. These stimuli have been widely used to up-regulate FasL surface expression (14). In contrast to the perforin-mediated killing activity, Fas-mediated killing was partially restored by this treatment, suggesting that calcium is needed for the TCR signal-induced surface expression of FasL, but not for the interaction of the ligand with its receptor.

The Fas-based pathway kills targets with a lag period

Perforin-dependent killing is a rapid event due to the production and storage of readily accessible cytotoxic effector molecules prior to target cell contact. In order to compare the kinetics of perforin- and Fas-dependent killing pathways, target cells were incubated with *gld*-derived or perforin-

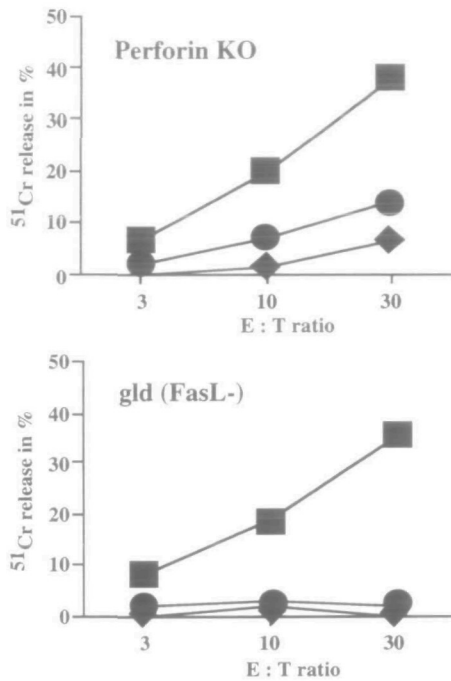


Fig. 1. EGTA inhibits Fas- and perforin-mediated cytotoxicity. The cytotoxic activity of MLC (5 days after stimulation) derived from perforin-deficient and *gld* mice was assessed in a standard 4 h ^{51}Cr -release assay against A20 target cells in the absence (squares) or presence (diamonds) of Mg^{2+} -EGTA. Effector cells were also preactivated for 3 h with PMA plus ionomycin and then assayed for their cytolytic activity (circles). E, effector, T, target.

deficient CTL at a 30:1 effector:target cell ratio (Fig. 2). At the indicated time points, membrane and DNA damage was measured. Hematopoietic target cells, i.e. A20 B lymphomas and Con A blasts, were rapidly killed via the perforin-mediated pathway, leading to significant membrane and DNA damage within the first 2 h (Fig. 2A). In contrast, killing by Fas showed a lag period of up to 2 h during which only a minor fraction of targets were killed. In particular, membrane damage was significantly delayed. Even at the endpoint of the assay (4 h), where both mechanisms showed comparable efficiency in DNA fragmentation, Fas-induced membrane damage was still inferior to perforin-mediated cytolysis (30–50% of reduction). Similar results were obtained when fibroblast lines were used as target cells (Fig. 2B). Again, the perforin-dependent pathway induced cell death more rapidly, although differences in membrane damage were less pronounced for 3T3.A31 target cells.

Fas-mediated cytotoxicity requires de novo RNA and protein synthesis

Perforin-mediated cytotoxicity is independent of *de novo* RNA and protein synthesis, since preformed cytotoxic effector molecules are already stored in cytoplasmic granules of activated T cells. To analyze the requirement for an intact metabolic machinery during Fas-mediated killing, cytotoxicity tests with A20 cells as targets were performed in the presence of either actinomycin D, an RNA synthesis inhibitor, or the

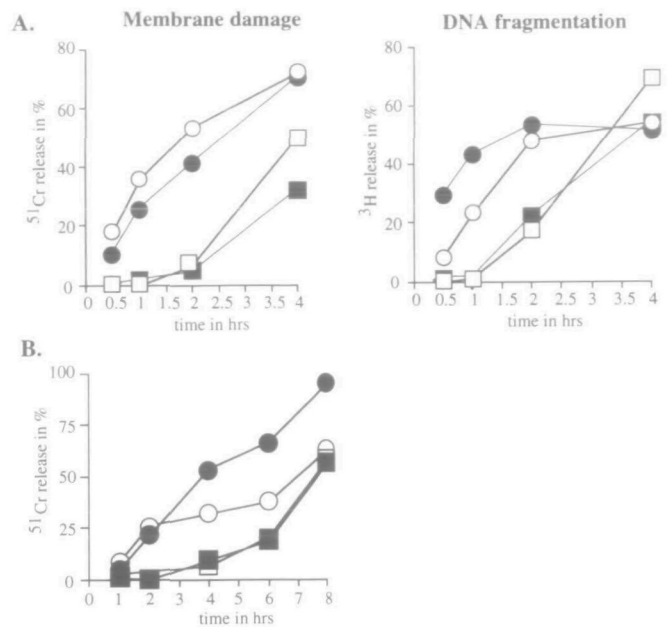


Fig. 2. Fas-mediated killing shows slower kinetics, but similar efficiency as the perforin-mediated pathway. (A) The cytotoxic activity of MLC derived from perforin-deficient (squares) and *gld* mice (circles) was measured in a standard 4 h ^{51}Cr - and ^3H thymidine-release assay against A20 cells (open symbols) and Con A blasts (filled symbols). Radioactivity released into the supernatant was measured at the indicated time points. (B) As in (A), but either WEHI-164 fibrosarcoma cells (filled symbols) or 3T3.A31 fibroblasts (open symbols) were used as target cells. ^{51}Cr release was measured at indicated time points up to 8 h.

two protein synthesis inhibitors cycloheximide and emetine. While perforin-mediated cytotoxicity was only slightly inhibited by either of the treatments (Fig. 3A), complete inhibition of Fas-mediated killing was observed, suggesting that both *de novo* RNA and protein synthesis is a prerequisite for this lytic activity. To demonstrate that the added inhibitors act on the effector and not on the target cells, perforin-deficient CTL were pretreated with either actinomycin D or emetine for 1 h and washed three times before testing their cytolytic activity. As shown in Fig. 3(B), the results were essentially the same as before, pinpointing the effect of the metabolic inhibitors to the effector cells.

Treatment of T lymphocytes with PMA plus ionomycin has been shown to increase FasL-specific mRNA and to up-regulate cell surface expression (14). Effector cells were incubated with these stimuli prior to the cytotoxicity test to investigate if such a pre-activation step could substitute for the need of *de novo* RNA and protein synthesis. After 3 h of incubation with PMA plus ionomycin, metabolic inhibitors were added and cytotoxic activity was determined. Killing activity of *gld*-derived CTL remained unchanged (Fig. 3C). In contrast, the actinomycin D-induced inhibition of Fas-mediated killing was rescued by the prior PMA plus ionomycin treatment. Continuous *de novo* protein synthesis, however, was still needed for efficient killing.

The critical requirement for ongoing protein synthesis during

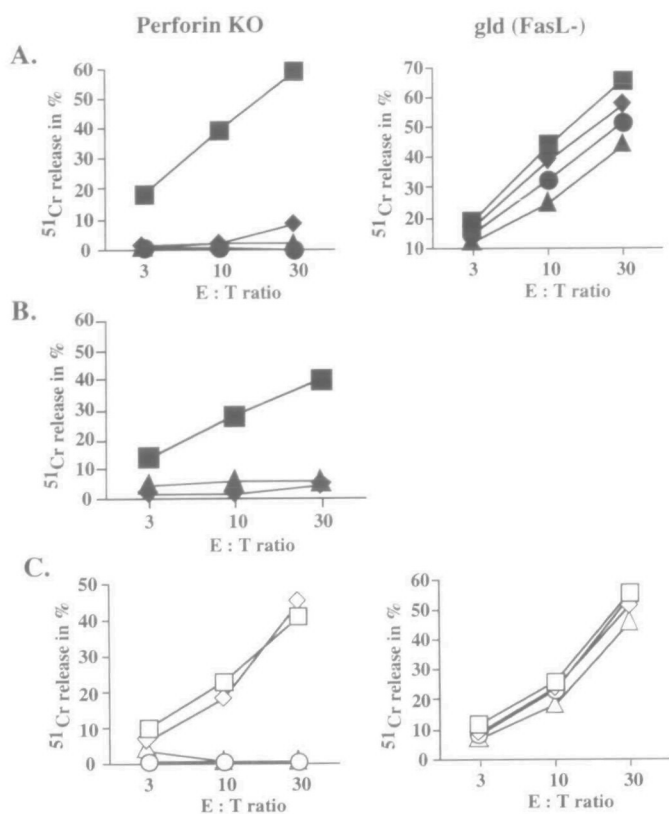


Fig. 3. Fas- and perforin-mediated killing can be discriminated by metabolic inhibitors. The cytotoxic activity of MLC derived from perforin-deficient and *gld* mice was assessed in a standard 4 h ^{51}Cr -release assay against A20 target cells in the absence (squares) or presence of actinomycin D (diamonds), cycloheximide (circles) and emetine (triangles). In (A), metabolic inhibitors were included during the whole assay. In (B), only effector cells were pretreated with the inhibitors for 1 h prior to the assay. In (C), effector cells were preactivated with PMA plus ionomycin for 3 h. Subsequently, the inhibitors were added and cytotoxicity assays were performed after 1 h of incubation. E, effector, T, target.

FasL-based killing was investigated in more detail. Although the direct interference of the protein synthesis inhibitors with FasL synthesis and surface expression remained the most likely explanation, other reasons such as reduced expression of adhesion molecules could not be excluded. Non-activated, activated or cycloheximide-treated, activated CTL were therefore analyzed by FACS for the presence of surface FasL. With reference on the isotype control, only a minority of non-activated cells (4.3%) specifically expressed FasL on their surface, whereas PMA plus ionomycin treatment up-regulated FasL surface expression in a distinct subpopulation (12.1%) (Fig. 4). Pretreatment of MLC with cycloheximide prior to activation reduced FasL expression to base levels (5.4%), suggesting that this FasL down-regulation may be responsible for the observed inhibition of cytotoxic activity. Similar results were obtained when cells were pretreated with emetine (data not shown). These findings are in agreement with earlier data (23), which showed that primary MLC-derived T lymphocytes, although they weakly express FasL on their cell surface (Fig. 4), are not capable to kill Fas-positive bystander cells, but

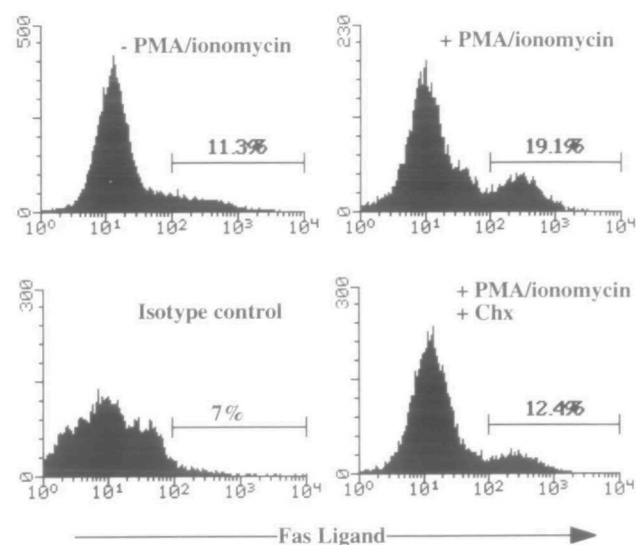


Fig. 4. Efficient FasL surface expression requires protein synthesis. MLC-derived lymphocytes were stained with an affinity-purified polyclonal antibody anti-FasL (anti-PE62) followed by donkey anti-rabbit coupled to FITC and analyzed by flow cytometry. Cells were analyzed after 4 h of incubation in the absence or presence of PMA plus ionomycin. In the last panel, cells were preincubated with cycloheximide for 1 h prior to activation. An isotype control was included.

need a second signal through TCR cross-linking. Our FACS experiments neglect a possible influence of Fas binding on FasL surface expression. Indeed, it has been shown for CD40L that interaction with its receptor leads to down-modulation of the complex. Thus, the half-life of FasL on a T lymphocyte may be even shorter in a 'CTL-target cell' than in a 'CTL only' system, explaining the dramatic effect of protein synthesis inhibitors on Fas-mediated cytotoxicity.

Primary MLC-derived T cells express intracellular FasL

In order to determine if the observed activation-induced surface expression is due to *de novo* synthesis of the ligand or rather due to translocation of the protein from an intracellular pool to the cell membrane, MLC enriched for T lymphocytes (B220⁻) were either analyzed by Western blotting or immunofluorescence microscopy using FasL-specific antibodies. Surprisingly, both methods revealed the presence of FasL already in MLC-derived T cells prior to TCR triggering. In Western blot analysis, a specific band with an apparent mol. wt of 40 kDa was detected, which could be competed with the specific (PE82, 100 $\mu\text{g}/\text{ml}$), but not with an unrelated peptide (PE58, 100 $\mu\text{g}/\text{ml}$) (Fig. 5). PMA plus ionomycin activation did not significantly change the level of FasL expression. However, caution must be taken in the interpretation of this experiment, since we observed considerable cell loss during the activation period. It may well be that activation leads to the induction of T cell suicide by the Fas pathway (2) and subsequently to the loss of FasL expressing cells. To localize FasL inside the cell, we performed immuno-staining of permeabilized non-

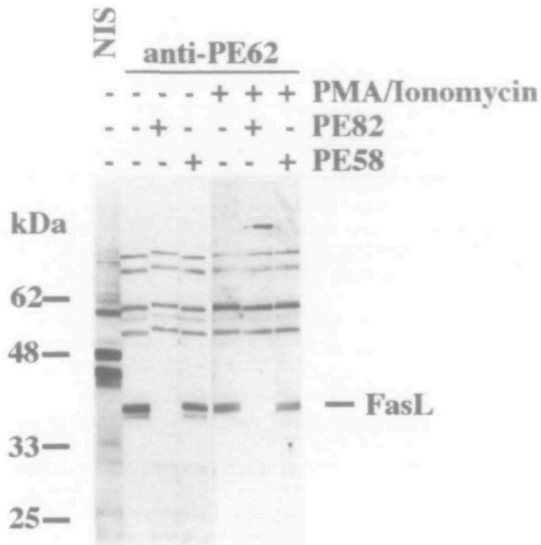


Fig. 5. MLC-stimulated T lymphocytes express FasL. Sorted MLC-derived T lymphocytes, cultured for 4 h in the absence or the presence of PMA plus ionomycin, were lysed and separated on a 10% SDS-PAGE under non-reducing conditions. Western blot analysis was performed using the affinity-purified polyclonal antibody anti-FasL (anti-PE62). As control, the non-immune serum (NIS) was included. To demonstrate the specificity of the anti-PE62 antibody, competition experiments were performed in the presence of either the specific (PE82; 100 μ g/ml) or a non-specific peptide (PE58, 100 μ g/ml). Note the presence of cross-reactive high molecular species, demonstrating equal loading.

activated T cells (Fig. 6A and D). The affinity-purified polyclonal antibody anti-PE62 demonstrated the presence of FasL within the cytoplasm as a distinct staining underneath the plasma membrane, which could be competed with the specific peptide PE82 (100 μ g/ml) (Fig. 6 B). Unfortunately, the sharpness of the staining was never high enough to detect any defined structure. In summary, our findings suggest that T cells accumulate intracellular FasL already upon allogeneic stimulation, but that the majority of the protein is only expressed on the cell surface upon a second TCR engagement.

Discussion

T cell-mediated cytotoxicity plays an important role in cellular immunity. Recently, two mechanisms have been shown to account for the main cytotoxic activity of *in vitro* and *in vivo* generated CTL and certain T cell lines (1,24-26). The 'classical' granule exocytosis pathway, strictly dependent on perforin, has been identified as one of the cytotoxic mechanisms. *In vivo* studies in perforin-deficient mice clearly showed that perforin-mediated cytotoxicity plays a major protective role in viral infections, immunity against bacteria (*Listeria*) and tumor rejection (2,26,27).

A second pathway involves the Fas/Apo-1 system (28). Fas-based killing was first reported to be active in the absence

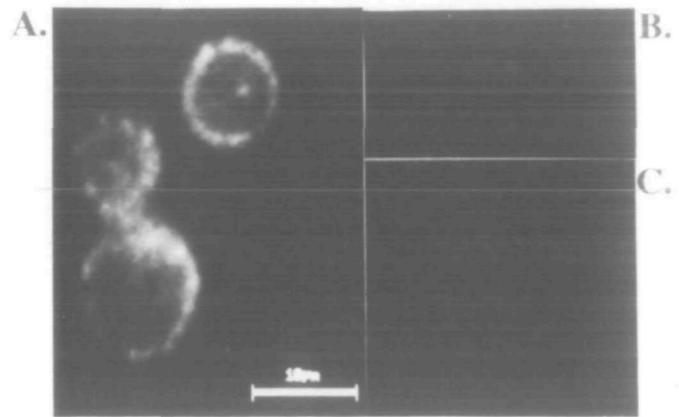


Fig. 6. MLC-stimulated T lymphocytes contain an intracellular pool of FasL. Sorted MLC-derived T lymphocytes were analyzed using confocal microscopy. Permeabilized cells were labeled with the affinity-purified antibody anti-PE62 either in the absence (A) or the presence (B) of the specific peptide PE82 (100 μ g/ml). (C) shows labeling with an isotype control.

of calcium (11), and, previously, EGTA has been used to discriminate perforin- and Fas-based pathways in CTL (11,29). In particular, unspecific killing induced by CTL clones or hybridoma cell lines clearly displayed Ca^{2+} -independent cytotoxic activity (30). However, recent studies of perforin-deficient T cell clones have provided controversial results (24). In our experimental system which studies specific target cell lysis by primary T cells, Fas-mediated killing strictly required extracellular calcium, most probably reflecting the TCR-dependent up-regulation of FasL on the cell surface. Even though FasL-Fas interaction is calcium-independent (31), the ion requirement for TCR signaling seems to dominate the specific Fas-killing pathway. The conflicting results in different systems may therefore be due to varying levels of constitutive FasL surface expression on the effector cells.

FasL belongs to the same protein family as tumor necrosis factor- α and lymphotoxin- α (14), two mediators of slow apoptotic cell death in sensitive targets (32). However, like perforin, FasL induces acute cytotoxicity. As demonstrated in this study, the kinetics of the two pathways are different. Whereas the perforin-based pathway leads to significant cell death shortly after target cell contact, Fas-dependent cytotoxicity is only effective after a lag-period of 2-3 h. TCR engagement has been shown to increase surface expression of FasL (13). Thus, the observed lag phase may reflect the need for sufficient FasL surface expression to ensure effective killing. Moreover, Fas-mediated cytotoxicity required continuous *de novo* protein synthesis which doubtlessly also contributes to the delayed onset of cytotoxicity. Metabolic inhibitors were used in earlier studies to discriminate between CD4⁺- and CD8⁺-mediated cytotoxicity (33,34). In contrast to CD8⁺ cells, CD4-mediated killing was strictly dependent on an intact metabolic machinery similar to our findings for the Fas pathway. Indeed, Fas-mediated cytotoxicity has been recently proposed to be the major killing pathway in CD4-restricted T lymphocytes (12). Although protein synthesis inhibitors inter-

with FasL surface expression, the question still remains open whether the drugs directly block the production of FasL or a presumed FasL-activating protein that is, for example, essential for membrane translocation or FasL oligomerization.

Our data show that a first allogeneic stimulation of T lymphocytes does not lead to pronounced cell surface expression of FasL, but rather to the synthesis of an intracellular FasL pool which is most likely recruited to the cell surface upon TCR triggering. This is reminiscent of CD40L which, upon B cell encounter, is rapidly translocated to the surface of T cells from intracellular stores. Prolonged surface expression of CD40L, however, also requires *de novo* synthesis and is inhibited by cycloheximide (35). The intracellular FasL store apparently does not suffice for efficient cell death induction. To date, the fate of FasL on the cell membrane before and after interaction with its receptor is not known. Due to FasL's potentially dangerous cytolytic activity, it is conceivable that surface FasL has only a short half-life and is either endocytosed and rapidly degraded or shed from the membrane. Our results agree with such a hypothesis, since the considerable quantities of FasL expressed on the cell surface upon PMA plus ionomycin activation are insufficient for efficient target cell lysis. The dependence on *de novo* synthesis of either FasL or a FasL-activating protein is also reflected by the decreased FasL surface expression after short exposure to cycloheximide (35). With regard to our results, it is interesting to note that CD40L surface expression is also tightly regulated. After TCR triggering, CD40L is only transiently expressed (36) and subsequent interaction with its receptor CD40 leads to down-modulation through receptor-mediated endocytosis (37). A similar mechanism may also be envisaged for FasL. Thus, FasL-Fas interaction upon effector-target cell contact would lead to continuous elimination of FasL, explaining the need for *de novo* protein synthesis. Given the dramatic effect seen after systemic delivery of anti-Fas antibodies to mice and the proposed role for the Fas/Apo-1 system in immune homeostasis, a tight regulation of FasL expression and activity may indeed be pre-mordial. Thus, it is tempting to propose that certain pathological situations may well result from a malfunction of such regulatory mechanisms.

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Abbreviations

Con A	concanavalin A
FasL	Fas ligand
Fas	Fas receptor
<i>gld</i>	generalized lymphoproliferative disease
<i>lpr</i>	lymphoproliferation
MLC	mixed lymphocyte culture
PMA	phorbol 12-myristate 13-acetate

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