Brief Definitive Report

Fas Involvement in Ca^{2+}-independent T Cell-mediated Cytotoxicity

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Summary

Mechanisms of T cell-mediated cytotoxicity remain poorly defined at the molecular level. To investigate some of these mechanisms, we used as target cells, on the one hand, thymocytes from *lpr* and *gld* mouse mutants, and on the other hand, L1210 cells transfected or not with the apoptosis-inducing Fas molecule. These independent mutant or transfectant-based approaches both led to the conclusion that Fas was involved in the Ca^{2+}-independent component of cytotoxicity mediated by at least two sources of T cells, namely nonantigen-specific in vitro activated hybridoma cells, and antigen-specific in vivo raised peritoneal exudate lymphocytes. Thus, in these cases, T cell-mediated cytotoxicity involved transduction via Fas of the target cell death signal.

Programmed cell death, often called apoptosis (1), massively occurs within the immune system as a component of its normal development (2–4). Part of this process seems to involve the Fas cell surface molecule (5–8), which can transduce a cell death signal (5, 6) and is not or is abnormally expressed (9) in the *lpr* mutant mouse (for review, see reference 10) exhibiting a lymphoproliferative disorder. Cell death can also be imparted by the immune system, in particular through T cell-mediated cytotoxicity. Hypotheses as to the undefined and apparently multiple molecular mechanisms of this process range from direct membrane interactions to granule exocytosis (for review, see reference 11). We demonstrate in this report a role for Fas in some instances of both nonspecific and antigen-specific Ca^{2+}-independent T cell-mediated cytotoxicity. Fas thus seems causally involved in cell death in both developmental and functional circumstances within the immune system.

Materials and Methods

Culture Conditions, Cells, and Cytotoxicity Test. All incubation and culture procedures were at 37°C in a water-saturated 5% CO_2 atmosphere. Cells (except PC60 and its derived clones) were grown in RPMI 1640 medium (Gibco Bio-Cult, Glasgow, Scotland) enriched with 10% FCS (Biological Industries, Beth Halmeck, Israel) and supplemented with 2×10^5 M 2-ME, penicillin, and streptomycin. PC60 is a hybridoma (12) between a mouse cytotoxic T cell clone with antimale D^b specificity (13) and a derivative from the rat T lymphoma W/Fu (C58NT)D. PC60 cells and all PC60-derived cells were grown and cloned in DMEM (Gibco Biocult, Uxbridge, UK) enriched with 5% FCS and the additives above. Cytotoxicity tests were carried out in V-shaped wells of 96-well microtiter plates with ^{51}Cr-labeled target cells (10^4 tumor cells or 10^5 thymocytes) and effector cells (PC60-derived d10S cells or peritoneal exudate cells [14]) at the indicated ratios in a total volume per well of 200 μl of RPMI medium with 10% FCS. The plates were centrifuged (200 *g*, 2 min) and incubated for 4 h at 37°C. After another centrifugation, 100-μl aliquots of supernates were assayed for radioactivity. The fraction of the total radioactivity released was then calculated, and the results were expressed as percent experimental ^{51}Cr-release − percent ^{51}Cr-release from target cells alone. d10S cells were incubated before and/or during the cytotoxicity tests with a mixture of PMA (Sigma Chemical Co., St. Louis, MO; final concentration 5 ng/ml) and the Ca^{2+} ionophore ionomycin (Calbiochem Corp., San Diego, CA; final concentration 3 μg/ml).

Mice. 4–6-wk-old MRL/Mp/Ola/Hsd and MRL/Mp-*lpr*/Ola/Hsd mice were obtained from Harlan Olac (Oxon, UK). C57Bl/6J-*lpr* or C57Bl/6J-*gld* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Other mice were bred at the Centre d'Immunologie.

Fas and Fas Transfectants. A cDNA spanning the entire coding region of the murine Fas antigen was obtained by reverse transcription (RT)-PCR using oligonucleotides corresponding to nucleotides 39–58 and 1294–1313 of the published sequence (8), flanked by EcoRI restriction sites to facilitate further cloning. 1 μg of poly(A)$^+$ B6 thymus mRNA was reverse transcribed using random hexamers (Pharmacia, Uppsala, Sweden) and Superscript mouse murine leukemia virus-H^- reverse transcriptase (Bethesda Research Laboratories [BRL], Gaithersburg, MD) for 1 h at 37°C. The reaction was incubated at 90°C for 5 min and 2 U of RNase H (BRL) were added. The reaction was further incubated for 20 min at 37°C. For PCR, 3 μl of the cDNA reaction was used with 50 pmol of phosphorylated oligonucleotide primers. The cycles were: 5 min at 94°C followed by 6 cycles (1 min, 94°C; 2 min, 55°C; and 3 min, 72°C) and 26 cycles (1 min, 94°C; 2 min, 60°C; and 3 min, 72°C), followed by 15 min at 72°C. 2.5 U of PFU polymerase (Strategene Inc., La Jolla, CA) was added after the annealing step of the first cycle. The PCR products were electrophoresed in Tris-acetate/EDTA buffer, an expected band of about 1,300 bp was

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excised from the gel, purified out of agarose, and digested with EcoRI (Boehringer, Mannheim, Germany) for 1 h at 37°C followed by 10 min at 65°C to inactivate the enzyme. This DNA was ligated with EcoRI-cut, calf intestine alkaline phosphatase-treated pBluescript II KS (−) vector (Stratagene Inc.) overnight at 15°C. The ligation mixture was then dialysed and electroporated into XL1 Blue bacteria (Stratagene Inc.). White colonies obtained after blue/white selection with XGal and isopropylthio-β-D-galactoside were analyzed for the presence of a 1,300-bp insert by PCR using T3 and T7 primers. Five "positive" clones were obtained, of which two were partially sequenced using a T7 sequencing kit (Pharmacia Fine Chemicals, Piscataway, NJ). Both clones were shown to contain both ends of the expected Fas sequence. Both inserts were digested out of the pBluescript II KS(−) vector with EcoRI, purified by electrophoresis and electroelution, cloned in the EcoRI site of a modified pHβAPr1-neo vector, and introduced in XL1 Blue. Two clones with the Fas cDNA correctly oriented towards the β-actin promoter were fully sequenced using internal primers. One of these clones, called pHβAPr1-neo-Fas 49, was identical to the published Fas sequence (8). Its DNA was prepared, purified by two CsCl gradients, and used to electrotransfect L1210 cells. After electroporation, the cells were seeded at 5×10^4–10^5 cells per well in 24-well microtiter plates and selected with G418 (1–1.5 mg/ml) (BRL). One of the resistant clones (called here L1210-Fas) was further tested for expression of Fas by Northern blots and for sensitivity to d10S incubated with PMA plus ionomycin (PI). Another round of transfection (data not shown) provided other L1210-Fas transfectants, some of which were also sensitive to d10S PI, whereas control vector-transfected L1210 cells were not.

Northern Blots. A volume of 10 μg of total RNA was electrophoresed per lane and blotted on nitrocellulose (Hybond N; Amersham Corp., Arlington Heights, IL). The membrane was prehybridized for 4 h at 42°C and hybridized with a Fas probe (6×10^5 cpm/ml) overnight at 42°C. The blot was washed twice at 65°C in 2× SSC-0.1% SDS and autoradiographed on Hyperfilm™ (Amersham Corp.).

Results and Discussion

Fas Involvement in a Model T Cell-mediated Cytotoxicity System. A convenient initial model was provided by the rat × mouse hybridoma PC60 obtained by Conzelmann et al. (12), which we serially cloned with systematic selection of the most cytotoxic clones (data not shown). The experiments described below used cells from the 10th serial subcloning, called PC60-d10S (d10S). Very similar results were obtained with later subclones. Whereas mixtures of interleukins induced the cytotoxic activity of PC60 in a matter of days (15), PI induced cytotoxicity much more readily. Effector d10S cells preincubated with PI for 3 h (d10S PI) or even subjected to PI only for the duration of the 4-h cytotoxicity test lysed, more efficiently than if untreated, YAC target cells (Fig. 1 *a*) and also thymocytes at particularly low E/T cell ratios (Fig. 1 *b*). Other characteristics (data not shown) of this cytotoxicity system were: (*a*) lysis was not MHC restricted (thymocytes of various MHC haplotypes, and the MHC-low YAC.5.2 cells [16] could be lysed) and could be inhibited in part by an anti-LFA-1 mAb antibody; (*b*) supernatants from d10S cells activated with PI were not lytic, even when highly concentrated, and even when collected from mixtures of effector and target cells; (*c*) cytolysis was not altered by addition of hexokinase or ATPase, N^G-monomethyl-L-arginine, acetate (thus implying no involvement of nitric oxide), an anti-TNF-α antiserum, or the lectin Con A; and (*d*) target cell death was accompanied by an apoptotic-type pattern of DNA fragmentation (17–19) and did not require macromolecular synthesis.

We considered the observation that thymocytes were sensitive to this type of cytotoxicity (Fig. 1 *b*) in the light of recent results showing that Fas is expressed on mouse thymocytes (8). To investigate whether Fas at the surface of target thymocytes might be causally involved in their lysis by d10S effector cells, we used thymocytes from *lpr* mutant mice, which do not express Fas (9). Whereas MRL thymocytes were sensitive (Fig. 1 *c*), MRL-*lpr* thymocytes were resistant (Fig. 1 *d*) to d10S cells, even in the presence of Con A (data not shown). The absence of lysis of *lpr* thymocytes by d10S cells was observed not only on the MRL, but also on the C57Bl/6

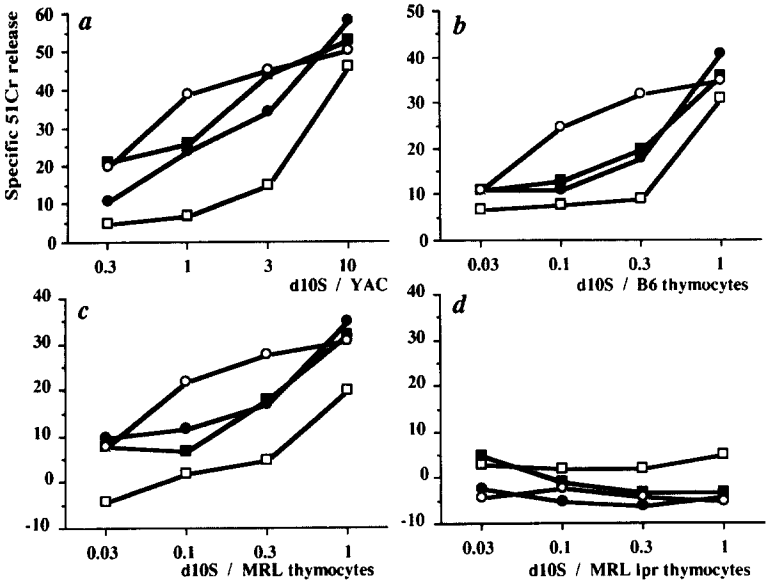

Figure 1. Cytotoxic activity of d10S cells. Target cells were either YAC cells (*a*) or thymocytes from C57Bl/6 (*b*), MRL/Mp/Ola/Hsd (*c*) and MRL/Mp-*lpr*/Ola/Hsd (*d*) mice. d10S cells (from the 10th serial subcloning of PC60) were induced to cytotoxicity by incubation with PMA and ionomycin, either for 3 h at 37°C before the cytotoxicity test (4×10^5 cells/10 ml of medium in a 75-ml tissue culture flask; pretest, ■), or by directly adding the mixture of PMA and ionomycin to the cytotoxicity test (in test, ●). In other groups both (○) or neither (□) induction procedures were followed. The results were expressed as percent experimental ^{51}Cr-release minus percent ^{51}Cr-release from target cells alone. In this experiment, the latter was 15–21%.

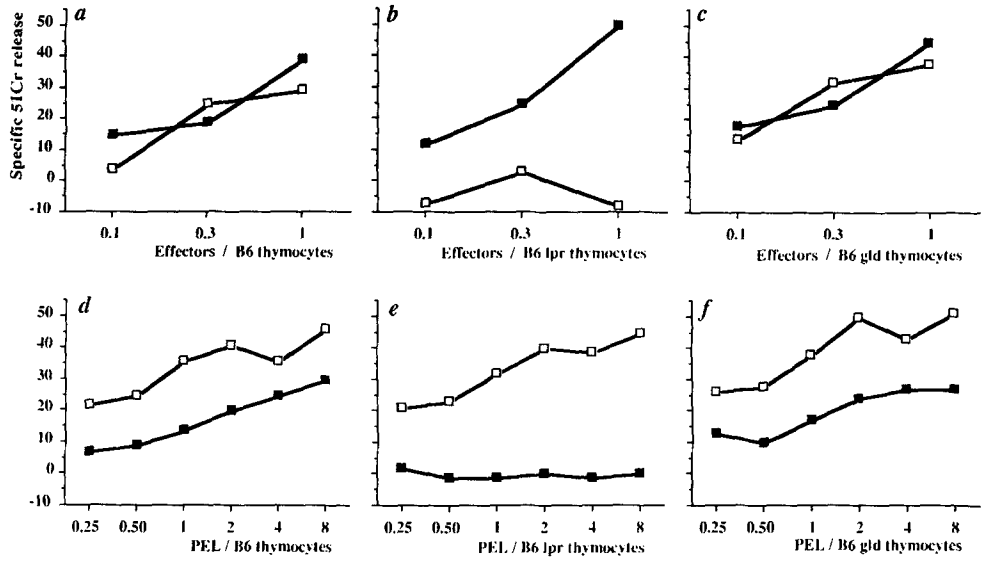

Figure 2. Differential sensitivity of thymocytes from wild type or mutant C57Bl/6 mice to d10S or PEL-mediated cytotoxicity. Target cells were thymocytes from 6–8-wk-old mice, either C57Bl/6 (*a* and *d*), or C57Bl/6J-*lpr* (*b* and *e*) or C57Bl/6J-*gld* (*c* and *f*). (*a–c*) Effector cells were either d10S PI (□), or *b* anti-*k* (between spleen cells from C3H, *k*, and C57Bl/6, *b*) 5-d MLC cells plus Con A (10 μg/ml final) in the cytotoxicity test (■); the latter cells were not cytotoxic to these thymocytes in the absence of Con A. In a separate experiment (*d–f*), effector cells were BALB/c anti-EL4 primary PEL sampled on day 11 and purified by two passages through nylon wool columns (final cell recovery 15%). The cytotoxicity test was performed either in complete medium (□) or in the presence of EGTA-Mg^{2+} (■). Spontaneous ^{51}Cr-release was 21–38%.

(Fig. 2 *b*) mouse backgrounds. Thus, thymocytes from mice not expressing Fas were resistant to d10S cells. In contrast, thymocytes from *gld* mice (20), which express Fas while having the same cellular phenotype as *lpr* mice (10) presumably due to the lack of the Fas ligand (21), were sensitive to d10S (Fig. 2 *c*). This indicated that *lpr* resistance was directly related to the absence of Fas at the target MRL-*lpr* thymocyte surface, not to a secondary consequence of this mutation on thymocyte development. In the presence of Con A, C57Bl/6-*lpr* thymocytes remained resistant to d10S (data not shown), but were nonspecifically lysed by MLC cells (Fig. 2 *b*), which provided a clearly Fas-unrelated positive lysis control. In line with the involvement in d10S-mediated lysis of the cell surface-expressed Fas molecule, the lysis by d10S cells of YAC cells and of MRL thymocytes was inhibited by MRL more than by MRL-*lpr* thymocytes, and also, in an MHC allele–unrelated manner, by C57Bl/6 more than by C57Bl/6 *lpr* thymocytes (data not shown).

To provide another, independent demonstration of the role of Fas as a target cell molecule transducing a cell death signal in some cytotoxicity systems, we converted an insensitive cell into a sensitive one by transfection with Fas. Although most of the 15 cell lines we tested, including YAC, EL4, and P815 (Fig. 3, *a–c*), were lysed by d10S PI, the L1210 lymphocytic mouse leukemic cell line was found insensitive to d10S PI (Fig. 3 *d*). Consistent with a requirement for Fas in the lysis by d10SPI, Fas transcripts were undetectable or abnormal in insensitive L1210 cells and *lpr* thymocytes, and were present in sensitive YAC cells and wild type thymocytes (Fig. 3 *f*). Transfection of Fas into L1210 conferred to these cells weak but definite Fas mRNA expression on the one hand (Fig. 3 *f*), and sensitivity to d10S PI on the other (Fig. 3 *e*). This line of evidence for the role of Fas in d10S PI-mediated cytotoxicity was independent from the one described above in that it involved L1210 cells rather than thymocytes, transfectants rather than mutants, and gain rather than loss of sensitivity. From another point of view, cytotoxicity was similar in the absence or presence of EGTA-Mg^{2+} (Fig. 3, *a–e*), showing the Ca^{2+} independence of d10S PI-mediated Fas-transduced cytotoxicity.

Fas Involvement in Ca^{2+}-independent Antigen-specific T Cell–mediated Cytotoxicity. The involvement of Fas was not limited to d10S PI-mediated cytotoxicity. It was also found for other antigen nonspecific PI–induced T cells (data not shown). To investigate antigen-specific cytotoxicity, we used peritoneal exudate lymphocytes (PEL), a model developed by Berke et al. (14) of potent specific in vivo-raised cytotoxic T cells. In view of the results with d10S PI, we especially considered the known Ca^{2+}-independent components (22, 23) of PEL-mediated cytotoxicity.

When tested on thymocytes, PEL showed (Fig. 2 *d*) two components of lysis, requiring or not requiring Ca^{2+}, respectively. Both of these components were antigen specific, since they lysed only (data not shown) the antigenically relevant thymocytes, in line with previous observations that the Ca^{2+}-independent components of MLC or PEL-mediated cytotoxicity were indeed antigen specific (22). However, the Ca^{2+}-dependent component lysed, and the Ca^{2+}-independent component did not lyse, *lpr* thymocytes (Fig. 2 *e*). Moreover, B6 *gld* thymocytes behaved like wild type, not *lpr*, thymocytes (Fig. 2 *f*), strongly indicating that the observed insensitivity of *lpr* thymocytes to Ca^{2+}-independent PEL-mediated cytotoxicity was directly related to the absence of Fas at their surface. As an independent further demonstration, when tested on tumor target cells, the Ca^{2+}-independent component of H-2d-specific PEL-mediated lysis (Fig. 4) spared L1210 (as observed before [23]) and YAC, and affected P815 and L1210-Fas. Thus, the two former cells, which lacked

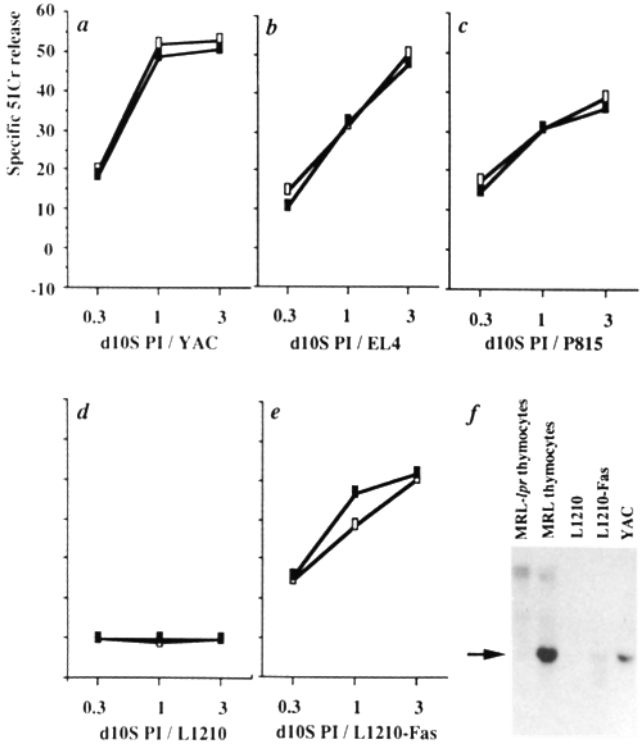

Figure 3. Sensitivity to d10S PI-mediated cytotoxicity of, and Fas expression in, YAC, EL4, P815, L1210, and L1210-Fas, i.e., L1210 transfected with Fas cDNA. The cytotoxicity test (*a–e*) was performed either in conventional medium (□) or in the presence of EGTA-Mg^{2+} (■). Spontaneous ^{51}Cr-release was 6–17%. A Northern blot of RNA from the indicated cells (*f*) was hybridized with a Fas probe. (*Arrow*) Migration of the main Fas transcript.

Fas (Fig. 3, *d* and *f*) or a recognized alloantigen (Fig. 4 *d*), respectively, were not lysed. The two latter, which bore both, could be lysed. All these results pointed to the requirement for antigen-specific Ca^{2+}-independent T cell–mediated cytotoxicity of both specific allorecognition and presence of Fas at the target cell surface.

This report provides models for cell-mediated cytotoxicity mechanisms involving molecular interactions between a ligand at the effector cell surface, and a receptor at the target cell surface (here, Fas) transducing a cell death signal. Another example may be macrophage or lymphocyte membrane-bound TNF (24–28) and TNF receptors, the latter being structurally related to Fas (7). Such mechanisms, making use of membrane-bound molecules related or not to those discussed here, would account for characteristics of cell-mediated cytotoxicity such as the polarity of lysis sparing the effector cells (29) and the apoptosis mode of target cell death (18, 19). d10S-mediated cytotoxicity requires the Fas molecule at the target cell surface, which signals an apoptotic cell death machinery within the target cell. This suggests that the killing machinery of the PI-induced d10S effector cell comprises a cell surface-expressed Fas ligand. Of practical interest, these cells may thus represent a good source material for the molecular cloning of this ligand. Alternatively, the effector cell ligand may recognize on the target cell surface not Fas but a molecule functionally associated with Fas. In any case, the same Fas molecule seems causally involved within the immune system in some types of cell-mediated cytotoxicity and in developmental cell death.

Antigen-specific Ca^{2+}-independent cytotoxicity would require in addition expression of the relevant TCR on effector cells and the relevant MHC on target cells. Thus, in this type of cytotoxicity, at the effector cell surface both the TCR and a Fas ligand would engage the corresponding target cell surface molecules, leading to antigen-specific, Fas-transduced target cell death. Hypothetically, at the surface of some cytotoxic T cells, the functional availability of a Fas ligand may be induced by antigen-specific engagement of the TCR, or by antigen nonspecific signals such as interleukins or phorbol ester and calcium ionophore. This would lead to antigen-specific or nonspecific lysis, respectively, of Fas-bearing target cells.

In vivo, Fas-based cytotoxicity pathways must be limited by expression of both a functional Fas ligand among effector cells and Fas among putative target cells. Fas expression has been reported, in mouse (8), in thymus, liver, ovary, and heart, but could not be detected in brain, spleen, bone marrow, kidney, testis, and uterus. In man (5–7, 30–32), Fas (identical to APO-1 [33]) was found expressed on various cell types such as myeloid cells and fibroblasts, on some but not all B and T lymphoid cell lines, on activated more than on resting

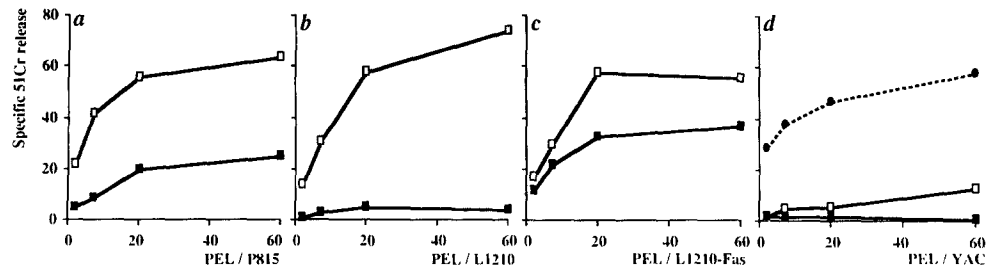

Figure 4. Differential sensitivity of tumor and transfectant cells to Ca^{2+}-independent antigen-specific PEL-mediated cytotoxicity. Effector cells were CBA anti-P815 primary PEL sampled on day 11 and purified by one passage through a nylon wool column (final cell recovery 27%). The cytotoxicity test was performed either in complete medium (□) or in the presence of EGTA-Mg^{2+} (■). Incubation in the presence of Con A provided a positive lysis control on YAC target cells (*dotted line*). Spontaneous ^{51}Cr-release was 8–16%.

lymphocytes, and on some virus-infected lymphocytes. It is interesting that the expression of Fas could be induced by IFN-γ (7, 8).

The involvement of Fas shown here accounts for only part (about 10–20% according to Figs. 2 and 4, consistent with previous estimates [22]) of specific T cell–mediated cytotoxicity, namely its Ca^{2+}-independent component. Fas-based mechanisms may well account for most of this component, at least with the target cells used here, since absence of Fas led to undetectable Ca^{2+}-independent cytotoxicity. In contrast, the Ca^{2+}-dependent mechanisms of cytotoxicity tested here did not require Fas. They may use other membrane molecules or altogether different lysis mechanisms involving for example, exocytosis.

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