Distinct T Cell Receptor Signaling Requirements for Perforin- or FasL-mediated Cytotoxicity

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Summary

A diverse array of signals are generated in a cytotoxic T lymphocyte (CTL) after the T cell receptor (TCR) engages the class I major histocompatibility complex (MHC) peptide complex. These signals result in a multitude of CTL effector functions, including cellular cytotoxicity, cell surface receptor expression, and cytokine secretion. We have examined signaling through the TCR in a wild type $CD8^+$, MHC-restricted, antigen-specific CTL clone, 14-7, and its interleukin 2–dependent variant clone 14-7FD. We report here that 14-7FD is unable to kill via the perforin mechanism of killing, yet is still able to kill via the Fas ligand/Fas mechanism and secrete interferon-γ in an antigen-specific manner. 14-7FD has cytolytic granules that contain perforin and serine esterases, which are secreted after phorbol ester and Ca^{2+} ionophore treatment. Lastly, to investigate which TCR signaling requirements were operational in 14-7FD, we examined TCR-triggered intracellular Ca^{2+} mobilization in the two clones. After TCR engagement, 14-7FD failed to mobilize intracellular Ca^{2+}, which may be the cause for its inability to trigger the perforin/granule exocytosis mechanism of killing. These results indicate that the signal transduction events that trigger perforin killing and the signaling requirements to induce FasL expression are distinct. We hypothesize that these two distinct TCR signal transduction requirements allow for separate activation of these two mechanisms of killing relating to their role in eradication of infected cells or regulation of immune responses.

For $CD8^+$ T cells, two cytolytic mechanisms have been defined at the molecular level (1–9). The perforin/granule exocytosis mechanism and the FasL/Fas mechanism are both activated after TCR engagement in a $CD8^+$ T cell (4, 6, 7, 10). The perforin mechanism is characterized by preformed perforin and serine esterase (granzymes), which are stored in cytolytic granules (11, 12) that are released and fused with the target cell membrane within minutes of CTL–target cell recognition (13, 14). Perforin may form holes in the plasma membrane of the target cell that could act as a conduit for the granzymes to enter and promote the cytoplasmic and nuclear damage characteristic of apoptosis (11, 12). Death of the target cell occurs quickly and is irreversible. Eventually the apoptotic cell lyses, which can be measured by ^{51}Cr release. In contrast to the expeditious killing of the perforin mechanism, the FasL/Fas mechanism of killing occurs more slowly. After the CTL recognizes the antigenic peptide–MHC complex, signals are transduced into the CTL to activate transcription and expression of FasL on the CTL surface, which can be blocked by transcription or protein synthesis inhibitors (2, 15). Once FasL is expressed on the surface, the CTL will lyse cells that express Fas (1, 2), and even other T cells (16–18), in a Ca^{2+}-independent manner. In addition to its involvement in target cell killing, the FasL/Fas interaction can mediate activation-induced cell death (18–21). Both target cell killing and antigen-induced cell death can be blocked with a Fas.Fc fusion protein that competes for FasL binding (9, 18, 22). Understanding why CTL have evolved these two mechanisms of killing and how they are activated and regulated is fundamental to understanding T cell–mediated effector function and regulation of immune responses.

To better understand the molecular mechanisms of CTL-mediated target cell killing, we examined the differences between a wild type cytolytic T lymphocyte, 14-7 (23), and its "noncytolytic" variant, 14-7FD (factor dependent)[1] (24). 14-7 is a $CD8^+$, K^d-restricted influenza virus (HA529-537)–specific T cell clone. 14-7 requires stimulation through the TCR, in the form of influenza-infected splenocytes, and exogenous IL-2 to proliferate. In contrast, 14-7FD no longer needs the competence signal generated through the TCR and requires only exogenous IL-2 for growth. Since 14-7FD could not kill influenza-infected P815 in a standard 6-h ^{51}Cr cytotoxicity assay, we decided to further compare

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[1] *Abbreviations used in this paper:* BLT, Nα-benzyloxycarbonyl-L-lysine thiobenzyl ester; CsA, cyclosporin A; ER, endoplasmic reticulum; FD, factor dependent; HA, hemagglutinin.

the differences between 14-7 and 14-7FD with regard to TCR-triggered effector functions. Reported here are comparative studies of granule exocytosis, perforin cytolysis, FasL/Fas–mediated cytolysis, and IFN-γ secretion.

Materials and Methods

Cells. CTL clones were established as described (25). Briefly, 14-7 was stimulated weekly in the presence of antigen (irradiated, influenza A/JAP/57–infected BALB/c spleen cells), 10 U/ml human rIL-2 (Biosource International, Inc., Camarillo, CA), 10% FCS, 2 mM glutamine, and 50 μM 2-ME in IMDM (GIBCO BRL, Gaithersburg, MD). The 14-7FD clone was passed biweekly in 30 U/ml rIL-2 in IMDM plus 10% FCS, 2 mM glutamine, and 50 μM 2-ME. L1210Fas+ (H-2^d) and L1210Fas$^-$ (H-2^d) (W. Clark, University of California at Los Angeles, Los Angeles, CA) and P815 (H-2^d) were used as target cells and were maintained in culture in DMEM (GIBCO BRL) supplemented with 10% FCS and 2 mM glutamine.

Intracellular Ca^{2+} Mobilization Studies. CTL clones were Ficoll purified 4 d after in vitro stimulation, and 10^6 cells/ml were incubated with 1 μM Indo-1 for 1 h and washed three times to remove free dye. CTL were stimulated with 90 μl of 1 μg/ml anti-CD3 mAb (145-2C11) or with 100 nM thapsigargin, and the ratio of 398:480 was measured over time. Excitation was at 340 nm in a spectrafluorometer (model 8000; SLM AMINCO, Urbana, IL) in the T format. Calibration was conducted as previously published (26).

RNase Protection Assay. Total cellular RNA was collected using RNAzol B (Biotecx Labs, Houston, TX). A 310-bp region of the mouse perforin cDNA (Genbank accession no. JO4148 nt 979-1289), generously provided by Pierre Henkart (National Institutes of Health), was inserted into the pGEM-3z vector (Promega Corp., Madison, WI) to provide a template for the perforin riboprobe. The template for the actin riboprobe was obtained from Ambion Inc. (Austin, TX). Antisense riboprobes were prepared using [α^{32}P]ATP and separated from unincorporated nucleotides using the Nuctrap push column (Stratagene Inc., La Jolla, CA). The hybridization protocol was performed using the RPA II kit (Ambion Inc.), and the reaction was allowed to continue for 16 h. 10 μg of each sample was loaded onto a 6% acrylamide/8 M urea sequencing gel. Electrophoresis was carried out at 250 V for 2.5–3 h, and the gel was dried at 50°C for 1 h. Protected fragments were detected on the gel using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), with an exposure time of 24 h.

Flow Cytometry. CTL were separated from dead cells and debris by isopaque–Ficoll centrifugation, and 5×10^5 cells were added to individual wells of a microtiter plate. Cells were stained on ice with 100 μl of either 145-2C11 (hamster anti–mouse CD3ε[27]), H57-597 (pan rat anti–mouse TCR-αβ [28]), FD441.8 (rat anti–mouse LFA-1 [29]), 7D4 (rat anti–mouse IL-2Rα[30]), 53.6-72 (rat anti–mouse CD8 [31]), and I3/23 (rat anti–mouse CD45 [from Matt Thomas, Washington University, St. Louis, MO]) hybridoma supernatants. After washing in PBS, bound antibody was detected with a FITC-labeled goat anti–hamster or goat anti–rat mAb (Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

IFN-γ ELISA. IFN-γ levels were determined on supernatants collected 4 h after stimulation. Supernatants were tested using a standard IFN-γ ELISA (PharMingen, San Diego, CA) as described (32).

BLT Esterase Assay. 96-well PVC round bottom plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 50 μl of 5 μg/ml mAb anti-CD3 (145-2C11), pan anti-TCR (H57-597), or anti-CD8 (53.6-72) overnight at 4°C. Plates were washed twice with PBS + 2% newborn calf serum before plating 14-7 and 14-7FD at 10^5 cells/well in quadruplicate. 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) and/or 1 μM ionomycin (Calbiochem Corp., La Jolla, CA) were added to the appropriate wells so that the total volume was 100 μl. The plate was spun for 2 min at 800 rpm before incubating the plate at 37°C for 4 h. 60 μl of the supernatants was harvested, and 20 μl was assayed for Nα-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterase activity as described (33), adapted for a 96-well microtiter plate, and the other 40 μl was used in an IFN-γ ELISA. After 30 min, absorbance values at 405 nm were determined by reading the plate on an EL-340 ELISA reader (Bio-Tek Instruments, Burlington, VT) at an OD of 405 nm. All experiments were performed in quadruplicate.

^{51}Cr Release Cytotoxicity Assay. Target cell lysis was measured by the ^{51}Cr release assay as described (34). Briefly, target cells were mock infected or infected with A/Jap/57 and loaded with sodium ^{51}chromate for 3 h at 37°C. Targets were washed three times and mock treated or sensitized with the HA529-537 (IYATVAGSL) peptide (0.1 μg/ml final) (23) before plating at 10^4 target cells/well. 14-7 and 14-7FD were added at an E/T ratio of 5:1, spun for 2 min at 900 rpm, and incubated at 37°C in a CO_2 incubator. After 6 h, 100 μl of supernatant was harvested from each well and counted on a gamma counter (Isomedic; ICN Biomedical, Huntsville, AL). All experiments were performed in quadruplicate. Spontaneous release was <10%. The percentage of specific lysis was calculated as follows:

$$\%\ \text{specific}\ ^{51}\text{Cr release} = 100 \times \frac{\text{experimental release} - \text{spontaneous release cpm}}{\text{total cpm} - \text{spontaneous release cpm}}$$

Results

14-7FD Is Unable to Effectively Lyse an Influenza-infected P815 Target Cell in a ^{51}Cr CTL Assay. To better understand the molecular mechanisms of T cell–mediated cytotoxicity, we examined the differences between a cytolytic CD8 T cell, 14-7 and its "noncytolytic" variant, 14-7FD. The CTL clone 14-7 is CD8$^+$, H-2 K^d restricted, specific for the transmembrane region of influenza virus hemagglutinin (HA 529-537)(23) and requires weekly antigen stimulation (A/Jap/57 influenza-infected splenocytes) and IL-2 for growth (24). A clone derived from 14-7, 14-7FD, is dependent on IL-2 for growth, but no longer requires antigenic stimulation to proliferate (24). In addition to not requiring TCR stimulation for growth, 14-7FD was unable to lyse influenza-infected P815 target cells in a standard 6 h ^{51}Cr release cytotoxicity assay (Fig. 1). As shown in Fig. 1, 14-7 lysed >70% of the A/Jap/57–infected P815 or peptide (HA 529-537) -pulsed P815, whereas 14-7FD lysed <10% of the A/Jap/57-infected or HA 529-537–pulsed P815 (Fig. 1). Since 14-7FD was noncytolytic in this 6 h assay, we wished to determine the nature of this defect in order to better understand the mechanism of TCR-triggered cytolytic effector function.

14-7 and 14-7FD Have Cytolytic Granules that Contain Perforin. Flow cytometric analyses revealed no significant

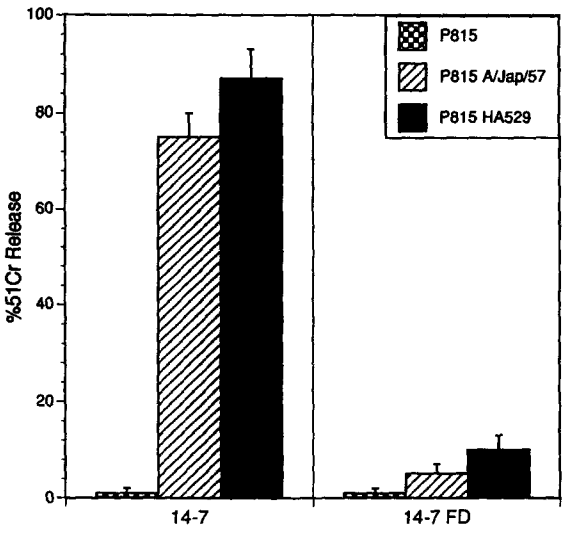

Figure 1. Cytolytic activity of 14-7 and 14-7FD on A/Jap/57 influenza-infected or HA 529-537-sensitized P815 target cells. Uninfected control P815 (*checkered bars*), A/Jap/57 influenza-infected P815 (*hatched bars*), HA 529-537 peptide (0.1 μg/ml)-sensitized P815 (*solid bars*) were used as target cells in a standard 6-h ^{51}Cr cytotoxicity assay.

differences in TCR, CD3, CD8, LFA-1, IL-2Rα, or CD45 levels between 14-7FD and parental 14-7 (data not shown). Since no gross deficits in surface expression of critical cell surface receptors involved in T-cell recognition and activation were detected, we examined whether 14-7FD had cytolytic granules that contained perforin and granzymes. Confocal microscopic and immunohistochemical evaluation revealed that 14-7FD has granules that are larger and more numerous than in 14-7 (Fig. 2). Interestingly, the granules are diffusely located throughout the cytoplasm in 14-7FD (Fig. 2 *b*), whereas they are localized to the tail region in 14-7 (Fig. 2 *a*). We also observed that 14-7FD is a rounder, more blastlike CTL than 14-7. Immunohistochemistry for perforin protein showed that both 14-7 and 14-7FD contain perforin within their granules (data not shown).

To confirm these studies, we determined whether 14-7 and 14-7FD could transcribe perforin mRNA after stimulation with plate-bound anti-CD3 mAb or with PMA and ionomycin using an RNase protection assay. Fig. 3 shows that unstimulated 14-7 had no detectable perforin mRNA, but did express perforin mRNA after stimulation by anti-CD3 or the combination of PMA and ionomycin. 14-7FD constitutively transcribed perforin mRNA and showed increased levels of perforin mRNA after stimulation via the TCR, PMA plus ionomycin, or ionomycin alone (Fig. 3). These data indicate that 14-7FD has cytolytic granules that contain perforin but is unable to execute perforin cytolysis after TCR stimulation.

Perforin/Granule Exocytosis in 14-7FD Can Be Triggered with PMA and Ionomycin But Not via the TCR. Functional analysis of perforin/granule exocytosis in 14-7 and 14-7FD was performed using the BLT esterase assay as a readout for TCR-triggered granule exocytosis (33). Examination of total levels of serine esterase activity in both clones revealed that 14-7FD has more than two times the amount of granzyme A as 14-7 (Fig. 4, *legend*). Since 14-7FD con-

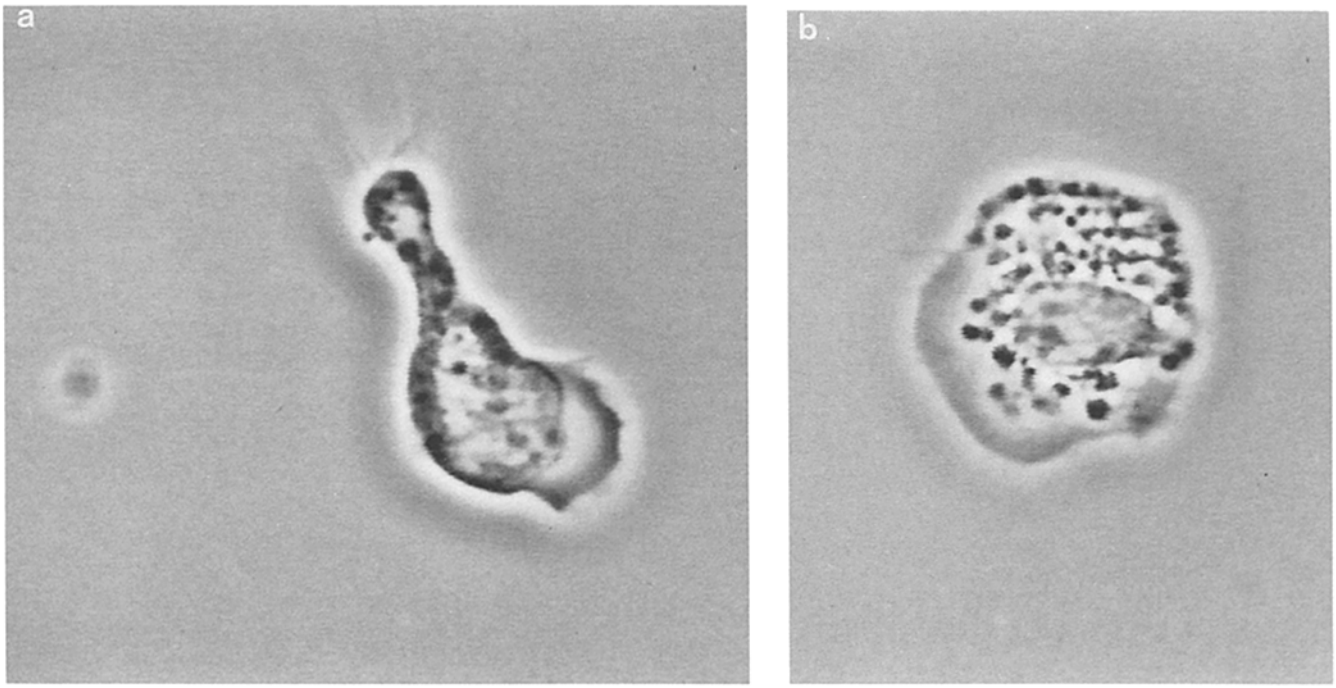

Figure 2. 14-7 and 14-7FD have cytolytic granules that contain perforin. Visualization of 14-7 (*a*), and 14-7FD (*b*), at ×250 magnification. Video microscopy was performed using a microscope (Microphot SA; Nikon Inc., Melville, NY) equipped with phase contrast and differential interference contrast optics housed in an environmental chamber maintained at 37°C. The camera's B/W signal was processed in real time on a digital signal processor (DSP2000; DAGE-MTI Inc., Michigan City, IN). ×250.

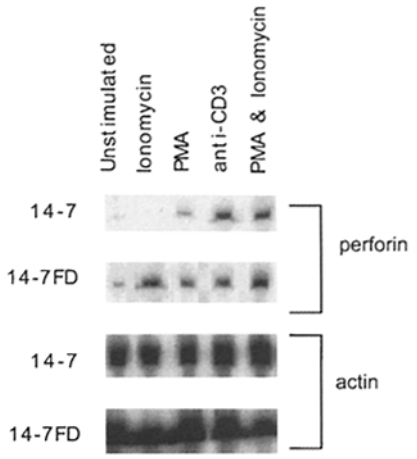

Figure 3. Expression of perforin mRNA in 14-7 and 14-7FD. Quiescent CTL, 7 d after stimulation, were stimulated with either anti-CD3 plate bound antibody (5 μg/ml), PMA (25 ng/ml), ionomycin (1 μM), or PMA and ionomycin. 16 h after stimulation, RNA from these cells was isolated. RNA samples were hybridized with either the actin or perforin DNA probes, separated, and detected as described in Materials and Methods. Protected perforin RNA was seen at a size of 310 bp, and actin control bands were seen at a size of 250 bp.

tained perforin and granzyme A, we examined whether it could secrete granzyme A after TCR engagement. TCR or CD3 cross-linking triggered granule exocytosis of granzyme A/BLT esterase was only observed in 14-7 and not in 14-7FD (Fig. 4). Previous studies have shown that PMA plus ionomycin can be used to bypass early TCR signaling

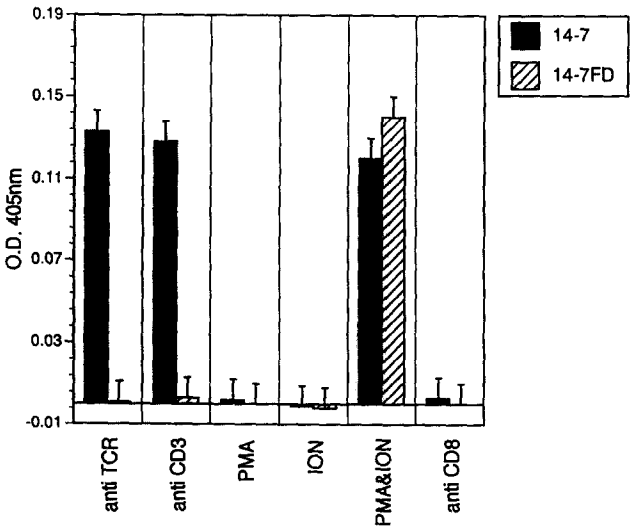

Figure 4. TCR, CD3, and PMA and ionomycin triggered perforin/granule exocytosis in 14-7 and 14-7FD. Detergent or total levels of BLT-esterase (granzyme A) were determined by lysing 10^5 CTL in 0.5% NP-40 in PBS. 14-7 (*solid bars*) or 14-7FD (*hatched bars*) were mock stimulated or stimulated with plate-bound anti-TCR mAb (H57-597), plate-bound anti-CD3 mAb (145-2C11), PMA and ionomycin (25 ng/ml PMA, 1 μM ionomycin), or plate-bound anti-CD8 (53.6-72) for 4 h, at which time culture supernatants were collected and assayed for BLT esterase activity. Values represent experimental values with spontaneous controls subtracted. BLT esterase absorbance at 405 nm for detergent lysed: 14−7 = 0.591, 14−7FD = 1.279; spontaneous release: 14-7 = 0.180, 14-7FD = 0.182.

events and trigger serine esterase release (33). PMA plus ionomycin treatment triggered granule exocytosis in both 14-7 and 14-7FD (Fig. 4). These results indicate that the perforin cytotoxicity defect was not due to an intrinsic inability to release the cytolytic granules.

Distinct TCR Signal Transduction Requirements Trigger Perforin Killing and FasL Induction after TCR Engagement. Other groups have reported that an alternate mechanism of killing used by T cells is mediated through FasL expression on the T cell and Fas on the target cell (1–9). Thus, we postulated that the 10% killing of influenza infected P815 by 14-7FD (Fig. 1) was mediated by FasL/Fas interactions. To test this possibility, we used a cell overexpressing Fas, L1210Fas^+ (1) and the same cell transfected with a Fas antisense vector, L1210Fas^- (2) pulsed with the antigenic peptide HA 529-537, as targets in a standard 6 h ^{51}Cr cytotoxicity assay.

14-7 killed both Fas^+ and Fas^- target cells that were sensitized with HA 529-537 (Fig. 5, *a* and *b*). In contrast, 14-7FD killed only HA 529-537–sensitized Fas^+ target cells (Fig. 5 *d*) and did not kill HA 529-537–sensitized Fas^- target cells (Fig. 5 *c*). It has been reported that induction of FasL in T cells is sensitive to protein synthesis inhibitors or cyclosporin A (CsA) treatment (4, 10, 15). Treating 14-7 with emetine or CsA has no effect on 14-7's ability to kill Fas^- (Fig. 5 *a*) or Fas^+ (Fig. 5 *b*) target cells, since 14-7 can still kill via the preformed perforin/granule exocytosis mechanism. In contrast, killing of HA 529-537–pulsed Fas^+ target cells by 14-7FD was inhibited by treating 14-7FD with emetine or CsA (Fig. 5 *d*), indicating that 14-7FD killed via the FasL/Fas mechanism only. This result also indicated that FasL induction may require calcineurin activation, as had been previously suggested (10). To further confirm 14-7FD was killing via the FasL /Fas mechanism, we tested if soluble Fas.Fc protein would inhibit killing. Soluble Fas.Fc totally inhibited 14-7FD's ability to kill HA 529-537–sensitized Fas^+ target cells (Fig. 5 *d*), whereas it had no effect on 14-7's ability to kill either Fas^- (Fig. 5 *a*) or Fas^+ (Fig. 5 *b*) target cells, since it could still kill via the perforin mechanism. At lower E/T ratios, the FasL component of 14-7's killing was apparent using emetine, CsA, or the Fas.Fc (data not shown). These results provide additional evidence that the signal transduction events that trigger perforin/granule exocytosis are not functioning in 14-7FD, whereas the signal transduction events that induce FasL expression are still intact.

TCR-signaled IFN-γ Production Occurs in Both 14-7 and 14-7FD. Since it was apparent that at least one TCR-activated effector function was still induced in 14-7FD, we examined whether other downstream CTL effector functions were activated in 14-7FD after TCR engagement. Using the same supernatants from the serine esterase granule exocytosis experiment (Fig. 4) to assay for IFN-γ, we determined whether TCR engagement induced IFN-γ production in 14-7 and 14-7FD. We observed that TCR signaling in 14-7 and 14-7FD resulted in the production and secretion of equivalent amounts of IFN-γ (Fig. 6). Bypassing early TCR signaling events with PMA and ionomycin also resulted in IFN-γ production in both clones (Fig. 6).

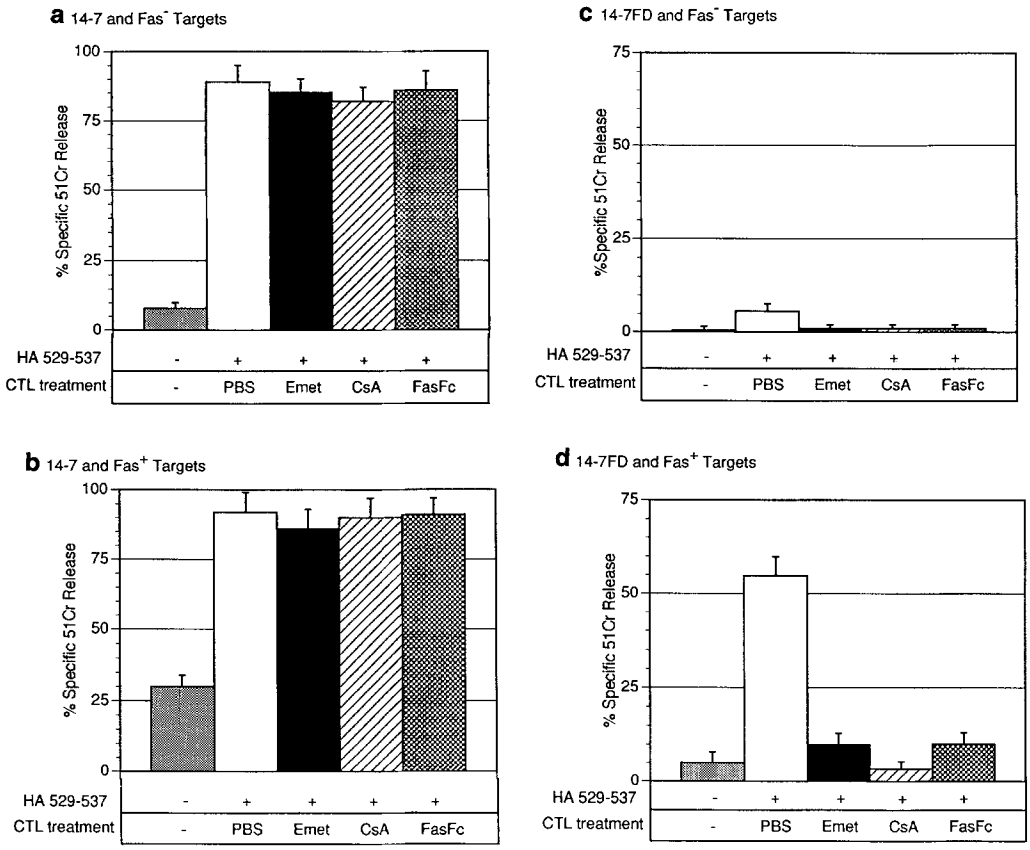

Figure 5. 14-7FD kills HA 529-537–sensitized Fas$^+$, but not HA 529-537–sensitized Fas$^-$ target cells, and its cytotoxicity is inhibited by emetine, CsA, or a Fas.Fc fusion protein. (*a*) 14-7 and L1210Fas$^-$ target cells; (*b*) 14-7 and L1210Fas$^+$ target cells; (*c*) 14-7FD and L1210Fas$^-$ target cells; (*d*) 14-7FD and L1210Fas$^+$ target cells. Untreated target cells (*shaded bars*) or HA 529-537 peptide–sensitized target cells (*open, solid, hatched,* or *checkered bars*) were used in a standard 6-h ^{51}Cr cytotoxicity assay. Emetine (1 μg/ml/10^{6}CTL) (*solid bars*) or CsA (5 μg/ml/10^6 CTL) (*hatched bars*) were used to pretreat the CTL for 2 h, and excess was removed by washing three times in MEM + 5% newborn calf serum (NBCS) before the start of the assay. 12.5 μg/ml of Fas.Fc fusion protein was added to the appropriate wells at the beginning of the 6 h incubation.

The detergent-lysed unstimulated T cells demonstrate no preformed IFN-γ in the unstimulated T cells. We also determined whether CsA would inhibit TCR-induced IFN-γ production in 14-7 and 14-7FD. A dose of CsA that had no effect on perforin killing by 14-7 completely blocked TCR-stimulated production of IFN-γ in both clones. These results provide more evidence that distinct signaling events are required to induce IFN-γ production and FasL expression versus those TCR signaling events required to execute perforin/granule exocytosis.

14-7FD Does Not Mobilize Intracellular Calcium after TCR Engagement. To determine whether abnormal TCR signaling events are responsible for 14-7FD's inability to kill via the perforin/granule exocytosis mechanism, we examined whether TCR-triggered changes in intracellular Ca^{2+} differ between the two clones. A TCR-triggered increase in intracellular Ca^{2+} is one of the earliest signaling events and occurs within seconds of TCR engagement. This TCR-stimulated increase in Ca^{2+} is characterized by a large increase of intracellular Ca^+ from endoplasmic reticulum (ER) stores, followed by a sustained influx of extracellular Ca^{2+}(35–38). Since an increase in TCR-triggered intracellular Ca^+ is required for granule exocytosis (26), we measured TCR-triggered Ca^{2+} mobilization and influx in the two clones. First, we observed that the resting levels of Ca^{2+} in 14-7FD (Fig. 7 *b*) are considerably higher than the resting Ca^{2+} levels in 14-7 (Fig. 7 *a*) (note the scale change between Fig. 7, *a* and *b*). We also found that stimulating 14-7FD with anti-CD3 (Fig. 7 *b*) failed to trigger the large characteristic increase in intracellular Ca^{2+} as seen in the parental 14-7 (Fig. 7 *a*). On closer examination of 14-7FD, we detected a small, but reproducible, increase in intracellular Ca^{2+}, possibly from extracellular sources (Fig. 7 *b*). A potential reason why 14-7FD could not initiate this large change in intracellular Ca^{2+} may be the lack of intracellular Ca^{2+} stores. To rule out this possibility, we used thapsigargin to inhibit an ER Ca^{2+} ATPase pump and thereby release ER Ca^{2+} into the cytoplasm (39). We observed that cytoplasmic Ca^{2+} levels increased in both 14-7 (Fig. 4 *c*) and 14-7FD (Fig. 4 *d*) after thapsigargin treatment. This result revealed that 14-7FD had ER Ca^{2+} stores, therefore excluding the possibility of a Ca^{2+} storage defect. Moreover, these results suggest that the reason 14-7FD can not kill via the perforin mechanism is due to a TCR signal

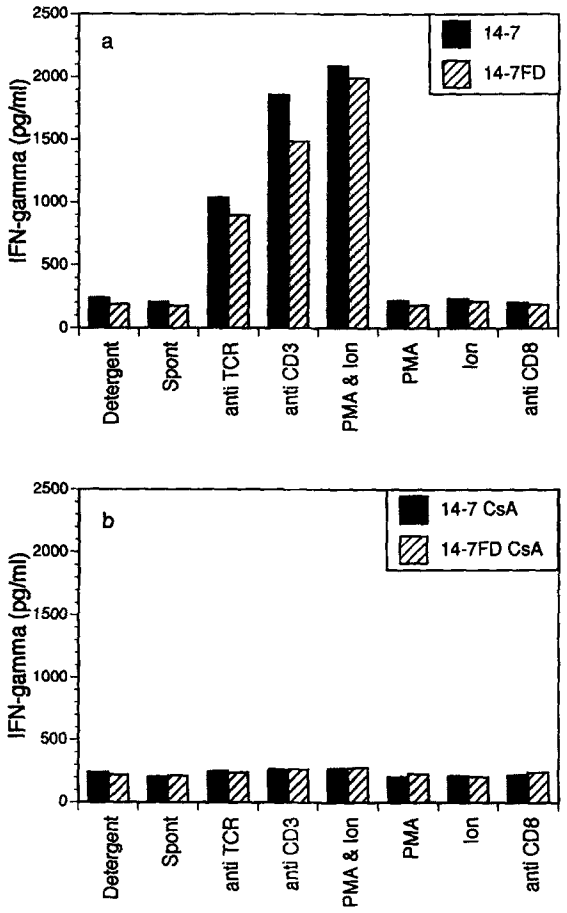

Figure 6. TCR/CD3 and PMA and ionomycin triggered IFN-γ production and secretion in 14-7 and 14-7FD. The culture supernatants from Fig. 5 (*a*) and from 14-7 and 14-7FD stimulated in the presence of CsA (5 μg/ml) (*b*) were assayed for IFN-γ content in an IFN-γ ELISA. 14-7 (*solid bars*) or 14-7FD (*hatched bars*). *Det*, levels of IFN-γ in unstimulated CTLs; *Spont*, amount of IFN-γ produced by CTL stimulated on PBS + 2% BSA–treated wells.

transduction event involved in releasing intracellular Ca^{2+}. Understanding this TCR-triggered Ca^{2+} mobilization defect and other signal transduction events involved in TCR-stimulated perforin killing and FasL induction warrants further investigation in 14-7 and 14-7FD.

Discussion

In this report we compared the cytolytic effector mechanisms of an antigen-dependent $CD8^+$ clone, 14-7, and an IL-2–dependent clone, 14-7FD, derived from 14-7. We sought to identify defects in the cytolytic mechanism or defects in signal transduction pathways that trigger the cytolytic mechanisms after TCR engagement. The comparison of these two clones has provided a model system for further characterization of intracellular signaling pathways that trigger perforin/granule exocytosis and induce FasL expression and IFN-γ production.

The cytolytic defect in 14-7FD was not due to the lack of TCR, CD3, CD8, or CD45 receptor expression, since the levels were comparable between 14-7 and 14-7FD. Although LFA-1 levels were lower on 14-7FD, no obvious deficit in expression was seen. Whether all the components of the TCR complex are functional in 14-7FD remains to be determined. In addition, 14-7FD was found to contain granules, perforin, and serine esterase (Fig. 2). Although 14-7FD could not kill via the perforin mechanism, it was able to produce IFN-γ and express FasL after signaling through the TCR (Figs. 5 and 6). TCR induction of IFN-γ production and FasL expression was blocked with either CsA or emetine, whereas CsA and emetine had no effect on the perforin mechanism of killing used by 14-7 (Figs. 5 and 6). PMA and ionomycin, used to bypass early TCR signal transduction events, triggered perforin/granule exocytosis in 14-7 and 14-7FD (Fig. 4). In addition to this result, the absence of a strong TCR-triggered Ca^{2+} flux in 14-7FD (Fig. 7) indicated that TCR signal transduction events that activate perforin/granule exocytosis are not functioning in 14-7FD, whereas distinct TCR signal transduction events that initiate FasL expression and IFN-γ production are still intact.

Our results with CD8 CTL and others with CD4 T cells (40) support a model in which the immune system can selectively interrupt TCR signal transduction through one biochemical pathway while preserving the functional capacity of others. To better understand the regulation of perforin and FasL/Fas killing, we are currently identifying which signal transduction pathways activate these two mechanisms of killing. The implications of having different TCR signal transduction pathways required for the perforin and FasL/Fas mechanisms of killing after TCR engagement are far reaching. The apparent biological roles of the perforin and FasL/Fas mechanisms of killing are very distinct. It appears that perforin killing is used by T cells to destroy parasitized cells that are infected during a pathogen invasion (5, 41), whereas the suggested primary role of the FasL/Fas mechanism of killing is downregulating an immune response by eliminating autoreactive T cells in the periphery (17, 42). Mice that are deficient in Fas(lpr) or FasL(gld) have lymphadenopathy, splenomegaly, produce large amounts of anti-DNA antibody and rheumatoid factor, and die of nephritis or arthritis at ~five mo of age (43-45). Expression of FasL must be tightly controlled because the presence of Fas on a number of tissues within and outside the immune system could make uncontrolled expression deadly (46). There is increasing evidence that disregulation of the FasL/Fas regulatory mechanism is occurring in rheumatoid arthritis, GVHD, superantigens (47), hepatitis C, and HIV (48). Interestingly, we have found that a foreign viral epitope activates the perforin mechanism of killing, whereas the homologous self-epitope can only activate the FasL/Fas mechanism of killing (49). This suggests that a self-peptide signals differentially through the TCR to selectively activate FasL expression without triggering perforin killing, in much the same way that TCR signaling initiates FasL expression without activating perforin/granule exocytosis in 14-7FD. In both cases, this selective TCR signaling could be used to modulate an immune response in the periphery.

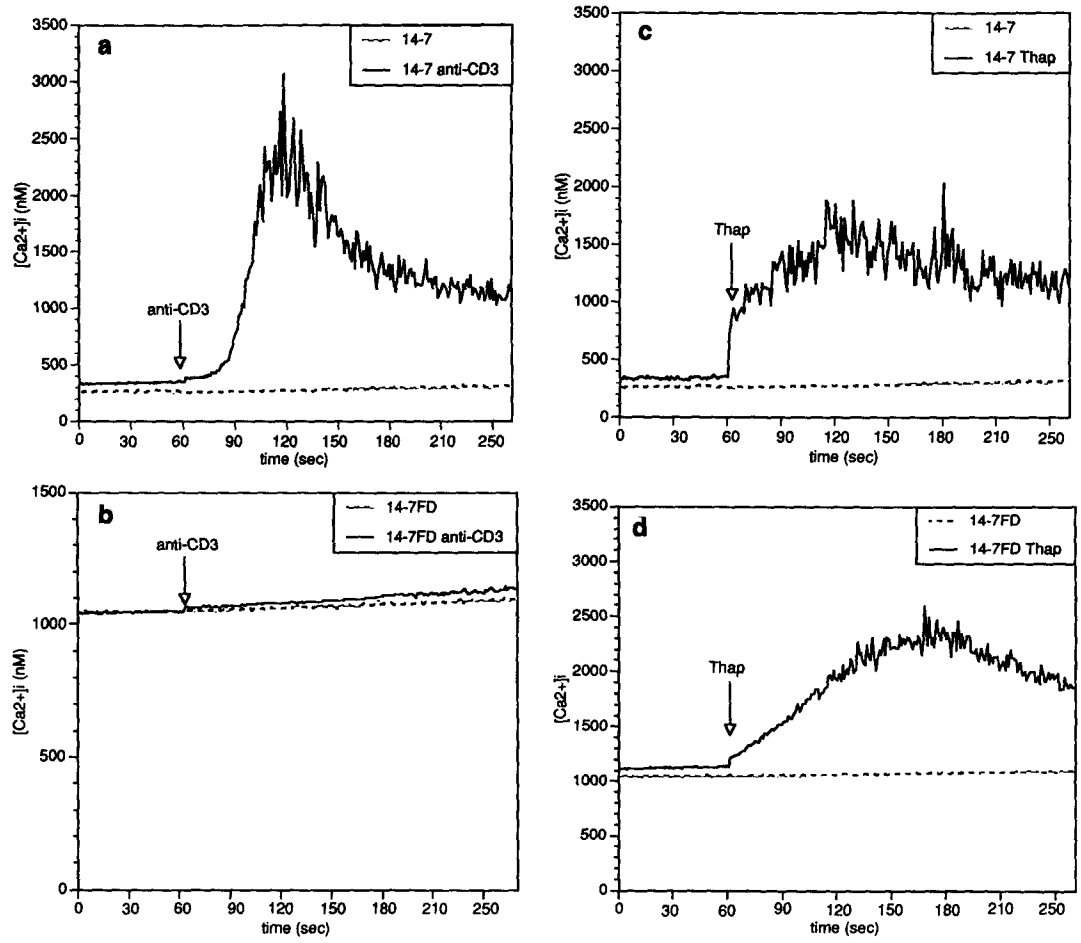

Figure 7. TCR/CD3–triggered intracellular calcium mobilization in 14-7 and 14-7FD. 14-7 and 14-7FD were mock stimulated with PBS, stimulated with anti-CD3 (90 μl of 1 mg/ml), or stimulated with thapsigargin (100 nM) and assayed for changes in intracellular calcium concentrations. (*a*) 14-7 control (*stippled line*) and 14-7 stimulated with anti-CD3 (*solid line*); (*b*) 14-7FD control (*stippled line*) and 14-7FD stimulated with anti-CD3 (*solid line*); (*c*) 14-7 control (*stippled line*) and 14-7 treated with thapsigargin (*solid line*); (*d*) 14-7FD control (*stippled line*) and 14-7FD treated with thapsigargin (*solid line*).

14-7 and 14-7FD provide a useful model to study TCR signal transduction in $CD8^+$ cytolytic T cells. 14-7FD does not require TCR stimulation for growth nor does TCR engagement trigger perforin/granule exocytosis. Therefore it was surprising that TCR engagement induced FasL expression and IFN-γ secretion without triggering Ca^{2+} mobilization in 14-7FD. It was surprising to find a T cell that did not require TCR stimulation for growth, wheras its TCR still possessed some signal transduction capabilities. TCR signal transduction is a complex issue since the antigen recognition complex contains 7 subunits, which contain a total of 10 immunoreceptor tyrosine activation motifs (ITAMs) (50, 51). The tyrosines within these motifs are phosphorylated by the src family kinases Lck and Fyn, which are activated within seconds of TCR engagement (52). Once phosphorylated, these tyrosines act as anchors for other SH2-containing signal transduction proteins such as Shc and ZAP70 (52). A possible explanation for 14-7FD's signaling defect is that only a subset of the tyrosines in 14-7FD's ITAMs get phosphorylated after TCR engagement, leading to only a subset of signals being transduced into the T cell, as has been shown with altered peptide ligands with CD4 T cells (53, 54). Another possibility is that one of these early-acting src family kinases or phosphatases involved in activating TCR signal transduction events is disregulated. This could be accomplished by a kinase or a phosphatase either being constitutively active or inactive. Studies to examine the role of the Fyn, Lck, and CD45 phosphatase in TCR-triggered perforin killing and induction of FasL in 14-7 and 14-7FD are in progress.

It is now apparent that multiple signaling pathways can emanate from a single receptor (55) and at least three distinct signal transduction pathways have been identified in T cells (40, 56–58). These include the phospholipase Cγ/Ca^{2+}/protein kinase C, PI3Kinase, and mitogen-activated protein kinase or MAP kinase signaling pathways (56). TCR engagement normally induces a rapid increase of intracellular Ca^{2+} ions followed by sustained elevated levels that are maintained for several minutes (26, 35, 37). Increased Ca^{2+} concentrations result in the activation of the calmodulin-dependent ser/thr phosphatase, calcineurin. Once activated, calcineurin dephosphorylates nuclear factor of activated T cells phosphoprotein, which translocates to the nucleus to induce gene transcription (59). CsA binds to

an immunophilin, which targets and inhibits the action of calcineurin (59). Thus it appears contradictory that 14-7FD does not have the characteristic Ca^{2+} mobilization profile yet is sensitive to CsA. On closer examination, there is a slight increase in $[Ca^{2+}]_i$ after TCR ligation (Fig. 7 *b*), which may be coming from another source other than the ER, such as Ca^{2+} channels in the plasma membrane, to activate calcineurin. It is noteworthy to point out that this is not the first system where this paradoxical relationship between CsA sensitivity and apparent lack of TCR-triggered Ca^{2+} flux has been observed. Anergic CD4 T cells that fail to produce IL-2, yet still produce IL-4 after TCR triggering, do not have a detectable increase in intracellular Ca^{2+} after TCR triggering (60, 61), but are still sensitive to CsA (40). It is worth noting that others have reported that CsA inhibits BLT esterase secretion (62), but only partially inhibits or does not inhibit CTL-mediated cytotoxicity (63–65). In addition, it has not been determined whether CsA inhibits the exocytosis of preformed BLT esterase or the production of newly synthesized BLT-esterase secreted through the constitutive secretion pathway (66). Studies using EGTA as well as Ca^{2+} channel blockers are in progress to clarify the role of intracellular and extracellular Ca^{2+} in TCR-signaled perforin killing and FasL/Fas killing.

In conclusion, we have shown that distinct signal transduction events are required to trigger perforin/granule exocytosis versus induce FasL expression and IFN-γ production. Our data suggests that distinct signal transduction pathways separate these two mechanisms of killing. Depending on the state of the T cell or the conformation of the Ag/MHC complex, TCR engagement can lead to IFN-γ production and FasL expression without perforin/granule exocytosis. These results support a model in which TCR signal transduction can differentially signal through one or several biochemical pathways. By modulating these TCR signal transduction pathways, the immune system could selectively execute (perforin) and downregulate (FasL/Fas) a cellular immune response.

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References

1. Rouvier, E., M. Luciani, and P. Golstein. 1993. Fas involvement in $Ca2^+$ independent T cell–mediated cytotoxicity. *J. Exp. Med.* 177:195–200.
2. Walsh, C., A. Glass, V. Chiu, and W. Clark. 1994. The role of the Fas lytic pathway in a perforin-less CTL hybridoma. *J. Immunol.* 153:2506–2514.
3. Henkart, P. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity.* 1:343–346.
4. Ju, S., H. Cui, D. Panka, R. Ettinger, and A. Marshak-Rothstein. 1994. Participation of target Fas protein in apoptosis pathway induced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. *Proc. Natl. Acad. Sci. USA.* 91:4185–4189.
5. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. Olsen, E. Podack, R. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature (Lond.).* 369:31–37.
6. Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science (Wash. DC).* 265:528–530.
7. Kojima, H., N. Shinohara, S. Hanaoka, Y. Someya-Shirota, Y. Takagaki, H. Ohno, T.S. Katayama, H. Yagita, K. Okumura, T. Saito et al. 1994. Two distinct pathways of specific killing revealed by perforin mutant cytototoxic T lymphocytes. *Immunity.* 1:357–364.
8. Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature (Lond.).* 370:650–652.
9. Hanabuchi, S., M. Koyanagi, A. Kawasaki, N. Shinohara, A. Matsuzawa, Y. Nishimura, Y. Kobayashi, S. Yonehara, H. Yagita, and K. Okumura. 1994. Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA.* 91:4930–4934.
10. Anel, A., M. Buferne, C. Boyer, V.A. Schmitt, and P. Golstein. 1994. T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A. *Eur. J. Immunol.* 24: 2469–2476.
11. Liu, C., C. Walsh, and J. Young. 1995. Perforin: structure

and function. *Immunol. Today.* 16:194–201.
12. Smyth, M., and J. Trapani. 1995. Granzymes: exogenous proteinases that induce target cell apoptosis. *Immunol. Today.* 16:202–206.
13. Peters, P., H. Geuze, H. Van der Donk, J. Slot, J. Griffith, N. Stam, H. Clevers, and J. Borst. 1989. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *Eur. J. Immunol.* 19:1469–1475.
14. Zagury, D., J. Bernard, N. Thierness, M. Feldman, and G. Berke. 1975. Isolation and characterization of individual functionally reactive cytotoxic T lymphocytes: conjugation, killing and recycling at the single cell level. *Eur. J. Immunol.* 5: 818–822.
15. Vignaux, F., E. Vivier, B. Malissen, V. Depraetere, S. Nagata, and P. Golstein. 1995. TCR/CD3 coupling to Fas-based cytotoxicity. *J. Exp. Med.* 181:781–786.
16. Vignaux, F., and P. Golstein. 1994. Fas-based lymphocyte-mediated cytotoxicity against syngeneic activated lymphocytes: a regulatory pathway? *Eur. J. Immunol.* 24:923–927.
17. Singer, G., and A. Abbas. 1994. The Fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity.* 1:365–371.
18. Alderson, M., T. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. Schooley, R. Goodwin, C. Smith, F. Ramsdell, and D. Lynch. 1995. Fas ligand mediates acitvation-induced cell death in human T lymphocytes. *J. Exp. Med.* 181:71–77.
19. Brunner, T., R. Mogil, D. LaFace, N. Yoo, A. Mahboubi, F. Echeverri, S. Martin, W. Force, D. Lynch, and C. Ware et al. 1995. Cell-autonomous Fas(CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature (Lond.).* 373:441–444.
20. Dhein, J., H. Walczak, H. Baumler, K. Debatin, and P. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1 (Fas/CD95). *Nature (Lond.).* 373:438–441.
21. Ju, S.T., D. Panka, H. Cui, R. Ettinger, M. el-Khatib, D. Sherr, B. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95) FasL interactions required for programmed cell-death after T-cell activation. *Nature (Lond.).* 373:444–448.
22. Cheng, J., T. Zhou, C. Liu, J. Shapiro, M. Brauer, M. Kiefer, P. Barr, and J. Mountz. 1994. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science (Wash. DC).* 263:1759–1762.
23. Braciale, T., M. Sweetser, L. Morrison, D. Kittlesen, and V. Braciale. 1989. Class I major histocompatibility complex-restricted cytolytic T lymphocytes recognize a limited number of sites on the influenza hemagglutinin. *Proc. Natl. Acad. Sci. USA.* 86:277–281.
24. Andrew, M., V. Braciale, and T. Braciale. 1984. Regulation of interleukin 2 receptor expression on murine cytotoxic T lymphocyte clones. *J. Immunol.* 132:839–844.
25. Braciale, T.J., M.E. Andrew, and V.L. Braciale. 1981. Heterogeneity and specificity of cloned lines of influenza virus–specific cytotoxic T lymphocytes. *J. Exp. Med.* 153:910–923.
26. Haverstick, D.M., V.H. Engelhard, and L.S. Gray. 1991. Three intracellular signals for cytotoxic T lymphocyte-mediated killing. *J. Immunol.* 146:3306–3313.
27. Leo, O., M. Foo, D. Sachs, L. Samelson, and J. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374–1378.
28. Kubo, R., W. Born, J. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine alpha beta T cell receptors. *J. Immunol.* 142: 2736–2742.
29. Sarmiento, M., D. Dialynas, D. Lancki, K. Wall, M. Lorber, M. Loken, and F. Fitch. 1982. Cloned T lymphocytes and monoclonal antibodies as probes for cell surface molecules active in T cell-mediated cytolysis. *Immunol. Rev.* 68:135–169.
30. Malek, T., R. Robb, and E. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA.* 80:5694–5698.
31. Ledbetter, J., and L. Herzenberg. 1979. Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63–90.
32. Schreiber, R. 1991. Measurement of mouse and human interferon-gamma. *In* Current Protocols in Immunology. J. Coligan, A. Kruisbeek, D. Marqulies, and W. Strober, editors. John Wiley & Sons Inc., New York. 6.8.1–6.8.8.
33. Takayama, H., G. Trenn, W. Humphrey, J. Bluestone, P. Henkart, and M. Sitkovsky. 1987. Antigen receptor triggered secretion of a trypsin-type esterase from cytotoxic T lymphocytes. *J Immunol.* 138:566–569.
34. Henkel, T.J., V.L. Braciale, and T.J. Braciale. 1987. Anti-T cell receptor antibodies fail to inhibit specific lysis by CTL clones but activate lytic activity for irrelevant targets. *J. Immunol.* 138:1221–1228.
35. Gray, L., J. Gnarra, and V. Engelhard. 1987. Demonstration of a calcium influx cytolytic T lymphocytes in response to target cell binding. *J. Immunol.* 138:63–69.
36. Imboden, J., and J. Stobo. 1985. Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. Exp. Med.* 161:446–456.
37. Nisbet-Brown, E., R. Cheung, J. Lee, and E. Gelfand. 1985. Antigen-dependent increase in cytosolic free calcium in specific human T-lymphocyte clones. *Nature (Lond.).* 316:545–547.
38. Ostergaard, H., and W. Clark. 1987. The role of Ca^{2+} in activation of mature cytotoxic T lymphocytes for lysis. *J. Immunol.* 139:3537–3579.
39. Thastrup, O., P. Cullen, B. Drobak, M. Hanley, and A. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.
40. Mueller, D., and M. Jenkins. 1995. Molecular mechanisms of underlying functional T-cell unresponsiveness. *Curr. Opin. Immunol.* 7:375–381.
41. Podack, E. 1995. Execution and suicide: cytotoxic lymphocytes enforce Draconian laws through separate molecular pathways. *Curr. Opin. Immunol.* 7:11–16.
42. Crispe, I. 1994. Fatal interactions: Fas-induced apoptosis of mature T cells. *Immunity.* 1:347–349.
43. Cohen, P., and R. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–269.
44. Singer, G., A. Carrera, A. Marshak-Rothstein, C. Martinez, and A. Abbas. 1994. Apoptosis, Fas and systemic autoimmunity: the MRL-lpr/lpr model. *Curr. Opin. Immunol.* 6:913–920.
45. Nagata, S., and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol. Today.* 16:39–43.
46. Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice.

Nature (Lond.). 364:806–809.

47. Ettinger, R., D. Panka, J. Wang, B. Stanger, S. Ju, and A. Marshak-Rothstein. 1995. Fas ligand-mediated cytotoxicity is directly responsible for apoptosis of normal $CD4^+$ T cells responding to a bacterial superantigen. *J. Immunol.* 154:4302–4308.
48. Westendorp, M., R. Frank, C. Ochenbauer, K. Stricker, J. Dhein, H. Walczak, K. Debatin, and P. Krammer. 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature (Lond.).* 375:497–500.
49. Cao, W., S.S. Tykodi, M.T. Esser, V.L. Braciale, and T.J. Braciale. 1995. Partial activation of $CD8^+$ T cells by a self-derived peptide. *Nature (Lond.).* 378:295–298.
50. Irving, B., and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell.* 64:891–901.
51. Weissman, A. 1994. The T-cell antigen receptor: a multisubunit signaling complex. *In* Lymphocyte Activation. L.E. Samuelson, editor. Karger, Basel, Switzerland. 59:1–18.
52. Peri, K., and A. Veillette. 1994. Tyrosine protein kinases in T lymphocytes. *In* Lymphocyte Activation. L.E. Samuelson, editor. Karger, Basel, Switzerland. 59:19–39.
53. Sloan-Lancaster, J., A. Shaw, J. Rothbard, and P. Allen. 1994. Partial T cell signaling: altered phospho-zeta and lack of Zap70 recruitment in APL-induced T cell anergy. *Cell.* 79:913–922.
54. Sloan-Lancaster, J., and P. Allen. 1995. Significance of T-cell stimulation by altered peptide ligands in T cell biology. *Curr. Opin. Immunol.* 7:103–109.
55. Heldin, C. 1995. Dimerization of cell surface receptors in signal transduction. *Cell.* 80:213–223.
56. Cantrell, D., M. Izquierdo-Pastor, K. Reif, and M. Woodrow. 1994. Signal transduction by the T-cell antigen receptor: regulation and function of p21ras and PtdIns-3 kinase. *In* Lymphocyte Activation. L.E. Samuelson, editor. Karger, Basel, Switzerland. 59:115–127.
57. Izquierdo-Pastor, M., S. Leevers, C. Marshal, and D. Cantrell. 1993. p21ras couples the T cell antigen receptor to extracellular signal-regulated kinase in T lymphocytes. *J. Exp. Med.* 178:1199–1208.
58. Izquierdo-Pastor, M., K. Reif, and D. Cantrell. 1995. The regulation and function of p21ras during T-cell activation and growth. *Immunol. Today.* 16:159–164.
59. Bierer, B. 1994. Cyclosporin A, FK506, and rapamycin: binding to immunophilins and biological action. *In* Lymphocyte Activation. L.E. Samuelson, editor. Karger, Basel, Switzerland. 59:128–155.
60. Gajewski, T., D. Lancki, R. Stack, and F. Fitch. 1994. "Anergy"of TH0 helper T lymphocytes induces downregulation of TH1 characteristics and a transition to a TH2-like phenotype. *J. Exp. Med.* 179:481–491.
61. Gajewski, T., D. Qian, P. Fields, and F. Fitch. 1994. Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. *Proc. Natl. Acad. Sci. USA.* 91:38–42.
62. Trenn, G., R. Taffs, R. Hohman, R. Kincaid, E. Shevach, and M. Sitkovsky. 1989. Biochemical characterization of the inhibitory effector of CsA on cytolytic T lymphocyte effector functions. *J. Immunol.* 142:3796–3802.
63. Lancki, D., B. Kaper, and F. Fitch. 1989. The requirements for triggering of lysis by cytolytic T lymphocyte clones. II. Cyclosporin A inhibits TCR-mediated exocytosis but only selectively inhibits TCR-mediated lytic activity by cloned CTL. *J. Immunol.* 142:416–424.
64. Strack, P., C. Martin, S. Saito, R. Dekruyff, and S. Ju. 1990. Metabolic inhibitors distinguish cytolytic activity of CD4 and CD8 clones. *Eur. J. Immunol.* 20:179–184.
65. Smyth, M., Y. Norihisa, and J. Ortaldo. 1992. Multiple cytolytic mechanisms displayed by activated human peripheral blood T cell subsets. *J. Immunol.* 148:55–62.
66. Isaaz, S., K. Baetz, K. Olsen, E. Podack, and G. Griffiths. 1995. Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a nongranule pathway. *Eur. J. Immunol.* 25:1071–1079.