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

By Mark T. Esser, Barath Krishnamurthy, and Vivian Lam Braciale

From the Department of Microbiology and the Beirne B. Carter Center for Immunology Research, University of Virginia, Health Sciences Center, Charlottesville, Virginia 22908

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 Ca2+ ionophore treatment. Lastly, to investigate which TCR signaling requirements were operational in 14-7FD, we examined TCR-triggered intracellular Ca2+ mobilization in the two clones. After TCR engagement, 14-7FD failed to mobilize intracellular Ca2+, 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 51Cr 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 Ca2+-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 FasFc 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+, Kd-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 51Cr cytotoxicity assay, we decided to further compare

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1Abbreviations used in this paper: BLT, N-α-benzoxycarbonyl-α-lysine thiobenzyl ester; CsA, cyclosporin A; ER, endoplasmic reticulum; FD, factor dependent; HA, hemagglutinin.

This work was presented in part at the Ninth International Congress of Immunology, San Francisco, CA, July 23-29, 1995.
the differences between 14-7 and 14-7FD with regard to TCR-triggered effector functions. Reported here are comparative studies of granule exocytosis, perforin cytolyis, 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 μM 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-2b) and L1210Fas− (H-2b) (W. Clark, University of California at Los Angeles, Los Angeles, CA) and P815 (H-2k) were used as target cells and were maintained in culture in DMEM (GIBCO BRL) supplemented with 10% FCS and 2 mM glutamine.

Intracellular Ca²⁺ Mobilization Studies. CTL clones were Ficoll purified 4 d after in vitro stimulation, and 10⁶ 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 chapsigargin, and the ratio of 398:480 was measured over time. Excitation was at 340 nm in a spectrofluorometer (model 8000; SLM AMINCO, Urbana, IL) in the T format. Calibration was conducted as previously published (26).

RNAase B Protection Assay. Total cellular RNA was collected using RNeasy B (Biotecx Labs, Houston, TX). A 310-bp region of the mouse perforin cDNA (Genbank accession no. J04148 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 [α²³P]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 30°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 × 10⁷ 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-2Ra [30]), 53.6–72 (rat anti–mouse CD8 [31]), and 13/23 (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).
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 51Cr cytotoxicity assay.

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 cytolyis after TCR stimulation.

Identification of 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-
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 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 perform 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 51Cr 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 e). 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-signal 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).
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 Ca²⁺ differ between the two clones. A TCR-triggered increase in intracellular Ca²⁺ is one of the earliest signaling events and occurs within seconds of TCR engagement. This TCR-stimulated increase in Ca²⁺ is characterized by a large increase of intracellular Ca²⁺ from endoplasmic reticulum (ER) stores, followed by a sustained influx of extracellular Ca²⁺ (35-38). Since an increase in TCR-triggered intracellular Ca²⁺ is required for granule exocytosis (26), we measured TCR-triggered Ca²⁺ mobilization and influx in the two clones. First, we observed that the resting levels of Ca²⁺ in 14-7FD (Fig. 7b) are considerably higher than the resting Ca²⁺ levels in 14-7 (Fig. 7a) (note the scale change between Fig. 7, a and b). We also found that stimulating 14-7FD with anti-CD3 (Fig. 7b) failed to trigger the large characteristic increase in intracellular Ca²⁺ as seen in the parental 14-7 (Fig. 7a). On closer examination of 14-7FD, we detected a small, but reproducible, increase in intracellular Ca²⁺, possibly from extracellular sources (Fig. 7b). A potential reason why 14-7FD could not initiate this large change in intracellular Ca²⁺ may be the lack of intracellular Ca²⁺ stores. To rule out this possibility, we used thapsigargin to inhibit an ER Ca²⁺ ATPase pump and thereby release ER Ca²⁺ into the cytoplasm (39). We observed that cytoplasmic Ca²⁺ levels increased in both 14-7 (Fig. 4c) and 14-7FD (Fig. 4d) after thapsigargin treatment. This result revealed that 14-7FD had ER Ca²⁺ stores, therefore excluding the possibility of a Ca²⁺ storage defect. Moreover, these results suggest that the reason 14-7FD cannot kill via the perforin mechanism is due to a TCR signal.
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-\(\gamma\) 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-\(\gamma\) and express FasL after signaling through the TCR (Figs. 5 and 6). TCR induction of IFN-\(\gamma\) 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-\(\gamma\) production are still intact.

Our results with CD8 CTLs 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(Ipr) or FasL(gld) have lymphadenopathy, splenomegaly, and 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.
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 Ca²⁺ mobilization in 14-7FD. It was surprising to find a T cell that did not require TCR stimulation for growth, whereas 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/ Ca²⁺/protein kinase C, PI3Kinase, and mitogen-activated protein kinase or MAP kinase signaling pathways (56). TCR engagement normally induces a rapid increase of intracellular Ca²⁺ ions followed by sustained elevated levels that are maintained for several minutes (26, 35, 37). Increased Ca²⁺ 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 \([\text{Ca}^{2+}]\), 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 the exocytosis of preformed BLT esterase or the ER, such as Ca\(^{2+}\) channels in the plasma membrane, to activate calcineurin. We thank Drs. William Clark and Craig Walsh for gift of the L1210Fas\(^+\) and L1210Fas\(^-\) cells, Dr. Pierre Henkart for the perforin probe, Ms. Noelle Williams for preparation of the perforin riboprobe, Dr. David Lynch for the generous gift of murine Fas.Fc fusion protein as well as critical reading of the manuscript, Dr. Doris Haverstick for assistance in the calcium fluorometry studies, and Scott Tykodi and Rhoel Dinglasan for helpful discussion.

This work was supported in part by the Beirne Carter Center for Immunology Research and grants from the U.S. Public Health Service to T. Braciale. M.T. Esser was supported by funds from U.S. Public Health Service training grant T32 CA 09109.

Address correspondence to Dr. Vivian Lam Braciale, University of Virginia, Beirne Carter Center for Immunology Research, HSC Box MR4-4012, Charlottesville, VA 22908.

Received for publication 13 November 1995 and in revised form 10 January 1996.

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