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Real-Time Detection of CTL Function Reveals Distinct Patterns of Caspase Activation Mediated by Fas versus Granzyme B

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Activation of caspase-mediated apoptosis is reported to be a hallmark of both granzyme B- and Fas-mediated pathways of killing by CTLs; however, the kinetics of caspase activation remain undefined owing to an inability to monitor target cell-specific apoptosis in real time. We have overcome this limitation by developing a novel biosensor assay that detects continuous, protease-specific activity in target cells. Biosensors were engineered from a circularly permuted luciferase, linked internally by either caspase 3/7 or granzyme B/caspase 8 cleavage sites, thus allowing activation upon proteolytic cleavage by the respective proteases. Coincubation of murine CTLs with target cells expressing either type of biosensor led to a robust luminescent signal within minutes of cell contact. The signal was modulated by the strength of TCR signaling, the ratio of CTL/target cells, and the type of biosensor used. Additionally, the luciferase signal at 30 min correlated with target cell death, as measured by a ⁵¹Cr-release assay. The rate of caspase 3/7 biosensor activation was unexpectedly rapid following granzyme B- compared with Fas-mediated signal induction in murine CTLs; the latter appeared gradually after a 90-min delay in perforin- or granzyme B-deficient CTLs. Remarkably, the Fas-dependent, caspase 3/7 biosensor signal induced by perforin-deficient human CTLs was also detectable after a 90-min delay when measured by redirected killing. Thus, we have used a novel, real-time assay to demonstrate the distinct pattern of caspase activation induced by granzyme B versus Fas in human and murine CTLs. *The Journal of Immunology*, 2014, 193: 000–000.

Cytotoxic T lymphocytes are essential to eliminate virus-infected cells and malignant cells in the body. CTLs kill target cells largely by one of two methods: 1) perforin-mediated granzyme delivery following exocytosis of cytotoxic granules, and/or 2) Fas ligand (FasL)-mediated coupling of target cell Fas death receptors. Immune synapse formation between CTLs and target cells triggers the rapid orchestration of the granule exocytosis pathway (1–3). At the immune synapse, granzymes enter the target cells following membrane perturbation by perforin. The

precise mechanism of how perforin delivers granzymes into the target cells is still under investigation (4–6). Granzymes are serine proteases, capable of cleaving numerous substrates in the cytoplasm and nucleus required for induction of apoptosis (7–9). Ten granzymes have been identified in the mouse and five in the human genome, suggesting redundancy. The targets of granzyme B proteolysis have been studied more extensively than the other granzymes. Direct cleavage and subsequent activation of caspase 3 by granzyme B is thought to be a critical first step in CTL-induced apoptosis.

Active caspase 3 is measurable in target cells by flow cytometry, representing one method by which CTL function may be assessed (10–13). Additionally, CTL function is routinely measured by loss of target cell membrane integrity or evidence of CTL degranulation by flow cytometry and/or by measurement of cytolysis by a gold standard, microplate assay of ⁵¹Cr release (14–16). Each of these approaches utilizes an end point assay. None of the current methodologies permit kinetic, continuous evaluation of cytotoxic function. Although perforin-mediated granzyme B delivery is described as an early event and Fas/FasL-induced death is described as occurring late, the only available technologies available to evaluate this activity in real time require sophisticated microchip technology (17). Additionally, monitoring of cytotoxicity by fluorescence-based approaches leads to a relatively low signal to noise ratio, due to background fluorescence from live or fixed cells. We reasoned that a protease-inducible, luminescent biosensor introduced into target cells alone would allow us to measure CTL-induced caspase induction in a living cell continuously, in a convenient, nonradioactive, microplate format with minimal background signal.

Firefly luciferase (*Photinus pyralis*) has been successfully engineered using circular permutation to be a protease specific

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J.L., S.K.F., M.B.J., A.C.A.V., B.L.B., B.F.B., and K.A.R. designed and executed experiments and interpreted data; A.F. and Y.T. generated the human cell lines from healthy adults and patients with perforin mutations; K.A.R. and B.F.B. designed the granzyme B-cleavable biosensors; and all authors contributed to the writing and review of the manuscript.

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Abbreviations used in this article: FasL, Fas ligand; GLS, GloSensor; HVS, herpesvirus saimiri; LCMV, lymphocytic choriomeningitis virus; RLU, relative light unit.

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biosensor, in which residues near the native termini are joined by a protease cleavage site. The permuted luciferase is sterically constrained and exhibits low levels of activity until the joining sequence is cleaved by the corresponding protease. Previous work has shown successful engineering of a caspase 3/7–cleavable biosensor, designed to detect the protease activity in cell-free lysates (18). We introduced a similar construct based on a thermal stable variant of *Photuris pennsylvanica* luciferase (GloSensor [GLS]) with a DEVD proteolytic site (GLS.DEVD) (19, 20) into EL4 target cells to detect intracellular caspase 3 activity following coincubation with CTLs in vitro. Within minutes of contact we observed a dramatic increase of luminescent signal. Importantly, the earliest activation of the biosensor was dependent on intact granzyme B and perforin, and the activity of the biosensor correlated with the gold standard cytotoxic function assay, ^{51}Cr release. Additional biosensors with granzyme B–cleavable luciferases yielded similar results, yet with distinctive kinetics in the early time period. In perforin-deficient murine and human CTLs, caspase activity was detectable after a 90- to 120-min delay and blocked by anti-FasL Ab, consistent with FasL/Fas-induced activity. Thus, we have created a nonradioactive assay capable of detecting the earliest changes in apoptosis, representing a major breakthrough to currently available CTL assays. We anticipate that this novel methodology will allow definition of genetic and pharmacologic modulators of CTL-mediated apoptosis in future studies.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6 wild-type and PRF1 $^{-/-}$ mice were obtained from The Jackson Laboratory and bred in our animal facility. P14 transgenic mice were obtained from P. Marrack (21). All murine studies were conducted in accordance with protocols approved by our Institutional Review Committee. Granzyme B cluster knockout mice were obtained from T. Ley (22). EL4 cells (American Type Culture Collection) were maintained in RPMI 1640 with 10% FBS. Firefly luciferase Ab was obtained from Abcam (Cambridge, MA). GLS cAMP reagent (biosensor substrate) was from Promega (Madison, WI). Etoposide was obtained from APP Pharmaceuticals. The diluent for etoposide included 0.2% citric acid, 3% benzyl alcohol, 8% polysorbate 80, 65% polyethylene glycol 300, and 30.5% (v/v) alcohol. Anti-FasL Ab (clone MFL4, human; clone MFL3, murine) was obtained from BioLegend and used at 100 $\mu\text{g}/\text{ml}$.

Constructs. The caspase 3/7 biosensor (GLS.DEVD) encoded by the pGLS-30F construct is a commercially available analog (GLS caspase 3/7, Promega) of the version previously described (18), based on a thermal stable variant of *P. pennsylvanica* luciferase evolved for enhanced detection of caspase 3 activity in living cells (19, 20). The plasmid containing the granzyme B/caspase 8 biosensor (GLS.IETD) was constructed by annealing the following oligonucleotides and ligating into the BamHI/HindIII sites of pGLS-30F plasmid: forward, 5'-GATCCGGCATCGAG-ACCGACAGCGGA-3', reverse, 5'-AGCTTCCGCTGTCGGTCTCGATGCG-3'. The oligonucleotides used to generate GLS.IEAD were as follows: forward, 5'-GATCCGGCCGGATCGAGGCCGACAGCGAA-3', and reverse, 5'-AGCTTTCGCTGTCGGCCTCGATCCGGCCG-3'. For GLS.VGPD, the oligonucleotides were forward, 5'-GATCCAAGAGCGTGGGCCCCGACTTCGGA3', and reverse, 5'-AGCTTCCGAAGTCGGGGCCACGCTCTTG-3'. All four biosensors were also cloned into a retroviral vector pMIEG, allowing detection of transduced cells using GFP expressed in *cis* (23). Oligonucleotide sequences used to generate control vectors may be obtained by contacting the authors.

In vitro testing of recombinant GLS

Recombinant GLS proteins were generated using the TNT T7-coupled wheat germ extract system (Promega) supplemented with the FluoroTect GreenLys in vitro translation labeling system (Promega). Following cell-free expression for 2 h at 30°C, aliquots of cell-free expression lysate were combined with an equal volume of murine granzyme B (Sigma-Aldrich) in assay buffer (100 mM HEPES [pH 7.4], 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA, 20% glycerol). Following a 1-h incubation at 37°C, 10 μl sample was combined with 90 μl luciferase assay reagent and luminescence was measured at room temperature on a GloMax-Multi+ luminometer (0.5 s integration time).

EL4 target cell transfection and cloning

EL4 and P815 cells were transfected with GLS caspase 3/7 or derivatives using FuGENE (Promega). Cells were selected and maintained with 600 $\mu\text{g}/\text{ml}$ G418 starting 3 d after transfection. Clones were obtained by limiting dilution. For experiments using retroviral constructs, EL4 and YAC cells were infected with biosensor-expressing retrovirus and then sorted for GFP-expressing cells 7 d later without antibiotic selection.

Generation of CTLs

Ex vivo CTLs were generated from wild-type and gene knockout C57BL/6 mice following lymphocytic choriomeningitis virus (LCMV) infection (i.p., 200 PFU LCMV-WE); mice were sacrificed 7 d postinfection and total splenocytes were harvested and used directly for CTL assay. In vitro CTLs were generated as previously described (24). Briefly, TCR transgenic P14 mice were sacrificed and splenocytes were harvested and stimulated with LCMV gp33–41 peptide (Anaspec) for 48 h, followed by expansion in IL-2-containing medium for 3–5 d before use. Murine NK cells were isolated from C57BL/6 mice using negative selection and cultured in murine IL-2 at 1000 U/ml for 7 d before testing function. Human cytotoxic T cells were generated from PBMCs from healthy controls or a perforin-deficient patient by transformation with herpesvirus saimiri (HVS) as previously described (25) and maintained in 200 U/ml human IL-2. Permission to use human cells for research was obtained according to protocols approved by our Institutional Review Board.

Biosensor CTL assay

CTLs were washed once with PBS (pH 7.4) and resuspended in growth medium without phenol red and supplemented with 20 mM HEPES and 10% FBS. Cells were plated into 96-well, round-bottom, white plates (Corning) in replicate wells at 50 $\mu\text{l}/\text{well}$. Biosensor-expressing EL4 targets were harvested and washed with PBS, followed by incubation with 2% (v/v in phenol red-free growth media) biosensor substrate at 37°C, 5% CO_2 for 30 min and added to effector wells at 50 $\mu\text{l}/\text{well}$ containing 5,000–20,000 cells. The total volume per well for the assay was 100 μl . Following centrifugation at 200 $\times g$ for 5 min, the plate was incubated at 37°C in GloMax-Multi+ luminometer (Promega) and luminescence was measured kinetically every 3 min with an integration time of 0.5 s. Each datum point represents the mean of four replicate wells. A similar protocol was used for testing NK function against YAC target cells. For the redirected killing assay, P815 cells were preincubated with OKT3 at 5 $\mu\text{g}/\text{ml}$ for 30 min and then coincubated with human T cells to trigger the biosensor.

^{51}Cr -release assay

A ^{51}Cr -release assay was performed as previously described (26). EL4 target cells were pulsed with 100 ng/ml of LCMV gp33–41 for 1 h prior to ^{51}Cr labeling. Effector and EL4 target cells (5000 cells/well) were coincubated for 4 h at 37°C. ^{51}Cr release into supernatant was counted on a 96-well LumaPlate (PerkinElmer) by TopCount NXT. Cytotoxicity is reported as the percentage of ^{51}Cr released to the supernatant according to the following calculation: [(test – spontaneous)/(maximum lysis – spontaneous)] $\times$ 100. Maximum lysis was determined by addition of 1% Triton X-100 (Sigma-Aldrich) to 5000 EL4 target cells.

Results

Protease biosensors allow detection of perforin-mediated granzyme B delivery by CTLs in real time

Based on previous work that demonstrated caspase 3 activation was dependent on perforin-mediated granzyme B delivery, we reasoned that a caspase 3/7 luciferase biosensor expressed in target cells would detect the earliest events of CTL-induced apoptosis. The caspase 3/7 biosensor GLS.DEVD was constructed by insertion of a DEVD proteolytic site into a circularly permuted, thermal stable variant of *P. pennsylvanica* luciferase (Fig. 1A and Ref. 20). The GLS.DEVD has been validated in real-time kinetic assays to monitor caspase 3/7 activity within living cells, both in vitro and in vivo. We also designed three novel biosensors, GLS.IETD, GLS.IEAD, and GLS.VGPD, to be directly cleavable by granzyme B. The IETD cleavage site is a known target for both granzyme B and caspase 8 (27), and IEAD is also a known target for caspase 10 (28). Using ExpASY Peptide-Cutter, we confirmed that the recognition sequence for GLS.VGPD is only predicted to be cleaved by granzyme B (29, 30).

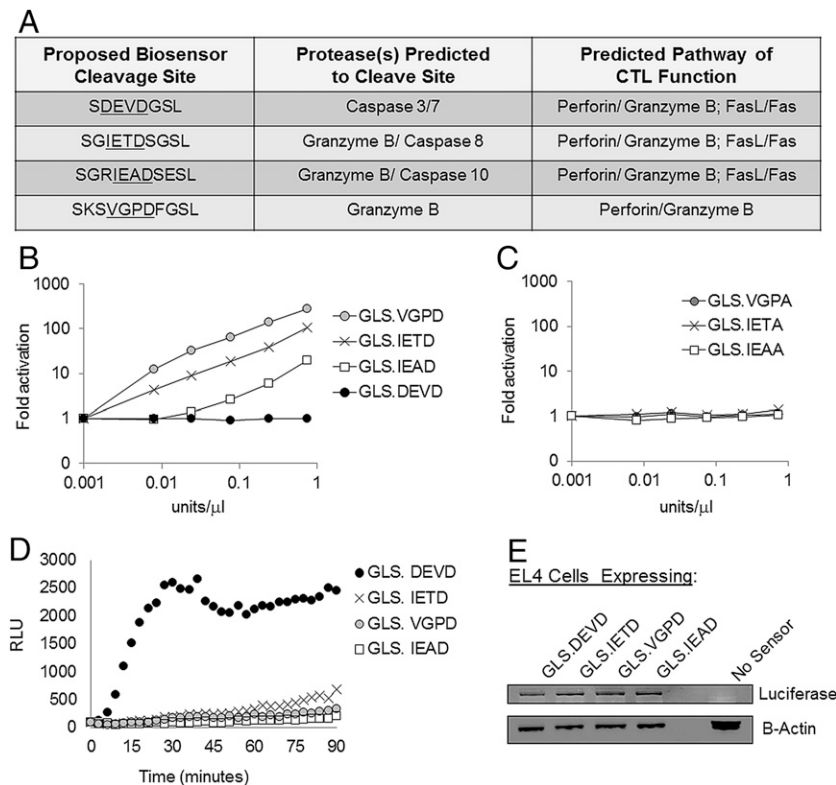


FIGURE 1. Design of caspase and granzyme B biosensors to measure CTL function. **(A)** We developed biosensors that directly measure granzyme B cleavage and/or caspase cleavage upon expression in target cells killed by CTLs. The biosensors include a circularly permuted luciferase containing a protease-cleavable site that allows activation of the luciferase only upon cleavage. Shown are the engineered protease cleavage site, the proteases predicted to cleave the site, and the pathways predicted to activate the biosensor following CTL/target cell recognition. **(B and C)** To demonstrate granzyme B dependence on the newly created biosensors, recombinant protein was generated in vitro and monitored for activation upon the addition of murine granzyme B. Granzyme B activated only the cleavage sites containing the required P1 aspartic acid residue (B), whereas the GLS.DEVD biosensor and control biosensors containing an alanine substitution for the P1 aspartic acid were inactive (B and C). **(D)** Biosensors were introduced into EL4 target cells by retroviral transduction, and cells were sorted for GFP expression. In vitro CTLs were generated by Ag (LCMV gp33–41) stimulation of splenocytes from P14 TCR transgenic mice. CTLs were coincubated with a biosensor-expressing EL4 target cells at E:T ratios of 6:1, and luciferase function was measured as the amount of RLU emitted. Data shown are from one cell line, representative of three independent experiments. **(E)** Protein lysates analyzed by Western blot using Abs recognizing luciferase and actin demonstrated equivalent biosensor expression in each cell line.

To confirm that the newly created biosensors would be activated by granzyme B, we generated recombinant protein using in vitro translation and tested for murine granzyme B cleavage across a range of concentrations (0.001–0.75 U/ μ l). Three additional control vectors were created, exchanging the critical aspartic acid within the proteolytic site with an alanine. As shown in Fig. 1B and 1C, neither the control vectors nor the GLS.DEVD was cleaved by granzyme B. The three novel biosensors were activated by murine granzyme B as shown in Fig. 1B. We predicted these novel biosensors would detect the earliest determinant of CTL function, granzyme B delivery into target cells, an event reported to precede activation of caspase 3 (7–9).

We introduced each biosensor into EL4 cells, a murine tumor cell line that is routinely used to present peptides in the context of H-2D_b. We generated stable lines by retroviral transduction followed by sorting of GFP-expressing cells of equal intensity to yield cell lines with nearly equivalent levels of luciferase protein (Fig. 1E). We first tested cytotoxic function of primary murine T cells cultured in IL-2 following Ag stimulation. These CTLs were derived from transgenic mice expressing a single TCR (P14) capable of recognizing LCMV gp33 peptide (referred hereafter as in vitro P14 CTLs). The gp33 peptide was presented by EL4 cells expressing each of the four biosensors. Following coincubation of CTLs and EL4 at 37°C at an E:T ratio of 6:1, all four biosensors were activated within minutes by CTL. The data from a repre-

sentative experiment are shown in Fig. 1D and Supplemental Fig. 1. The GLS.DEVD biosensor yielded the most robust signal (nearly 30-fold induction over target cells alone), with the remaining biosensors yielding 10- to 15-fold (GLS.IETD), 5- to 7-fold (GLS.VGPD), and 3-fold (GLS.IEAD) signals above background (for background, see Supplemental Fig. 1). The maximum signal for GLS.DEVD peaked by 30 min, whereas the GLS.IETD, GLS.IEAD, and GLS.VGPD biosensor signals continued to rise during the 90-min window of measurement. Each datum point represents quadruplicate wells. See Supplemental Fig. 1 for visualization of error bars from the biosensors and target cells alone, removed from Fig. 1D and subsequent figures to decrease crowding.

In an effort to achieve a maximal signal-to-noise ratio, the two biosensors with highest signal, GLS.DEVD and GLS. IETD, were transfected into cells in a traditional expression vector, pF9A, and clonal cell lines were obtained by limiting dilution in antibiotic selection. A maximally active clonal line was selected for further studies (Supplemental Fig. 2, for GLS.DEVD). Experiments were replicated using noncloned cell cultures, but the signal was consistently highest using clonal lines. We also expressed the biosensors in an NK target cell (YAC cells) and measured activity using IL-2-activated NK cells from C57BL/6 mice, which yielded a similar pattern of activation for GLS.DEVD, GLS.IEAD, and GLS.IETD, but the signal was relatively weak (Supplemental Fig. 3). Therefore, we moved forward with studies restricted to CTLs.

Biosensor luminescent signals detect changes in CTL activation

We evaluated the capacity of the biosensor to measure ex vivo CTL function obtained from mice infected with LCMV. We isolated splenocytes 1 wk postinfection, at a time when LCMV-specific CTLs have expanded to 0.5–2% of the total splenocytes (31, 32). CTL function was measurable at days 7 and 8 postinfection at a total splenocyte/target cell ratio of 12:1, which equates to a CTL E:T ratio of ~0.1:1 with individual variation from mouse to mouse. The data from a representative experiment are shown in Fig. 2. Both biosensors were activated within minutes by LCMV-specific CTLs. LCMV-derived CTLs initiated a peak response of the GLS.DEVD biosensor by 60 min, whereas the GLS.IETD biosensor signal continued to rise during the 90-min window of measurement.

Epitope density on target cell MHC has been previously shown to influence the strength of the CTL response (33, 34). To further test the capacity of the protease biosensors to detect changes in early CTL function, we titrated the concentration of the Ag (gp33) loaded onto EL4 target cells from 100 to 0.1 ng/ml. We observed that the luminescent signal induced by ex vivo CTLs decreased proportionally to the concentration of gp33 used to pulse target cells in EL4 expressing either the GLS.DEVD or GLS.IETD biosensor constructs. The GLS.DEVD signal was detectable down to peptide concentrations ≥ 1 ng/ml, whereas the GLS.IETD signal was detectable above the machine background when the gp33 concentration was ≥ 10 ng/ml, likely due to the lower baseline signal generated by GLS.IETD. The limit of detection of the GLS.DEVD signal correlated well with ^{51}Cr assays performed in parallel, with cytotoxicity detectable from 1 to 100 ng/ml and absent at 0.1 ng/ml (not shown). The GLS.DEVD biosensor signal was attenuated by a lower stimulating peptide concentration both in rate of induction (fold activation/time) and maximum signal obtained. It is apparent that T cell engagement/activation occurs over 2 log concentrations of gp33 (1–100 ng/ml) with minimal attenuation of caspase 3/7 activation, accounting for the preservation of cytotoxicity in this peptide concentration range as well (data not shown).

CTL induction of biosensor luminescence correlates with ^{51}Cr -release assay of cytotoxic function

We predicted that the early proteolytic events detected by biosensor activation would correlate with CTL-induced cytolysis of target cells and thus be useful as a nonradioactive microplate assay to substitute for the gold standard, 4-h ^{51}Cr -release assay. To control for cell number, we compared the luminescent signal to the ^{51}Cr -release assay for in vitro-derived P14 CTLs. As is typically done

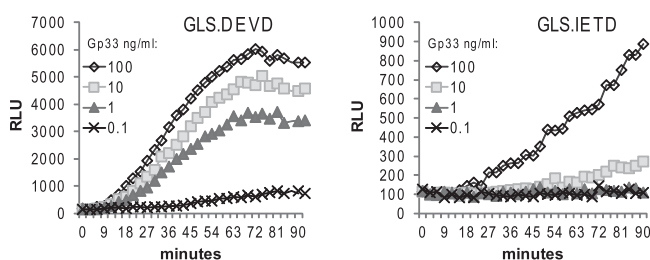


FIGURE 2. Strength of Ag recognition affects the induction of biosensors by ex vivo CTLs. Ex vivo CTLs from C57BL/6 mice infected with LCMV for 7 d were coincubated with luciferin substrate-loaded EL4 cloned target cells expressing GLS.DEVD or GLS.IETD. EL4 cells were pulsed with gp33 peptide at titrating concentrations and coincubated with ex vivo CTLs at a splenocyte/EL4 target ratio of 12:1. Luminescence (RLU) was measured every 3 min up to 90 min. Data are from one mouse, representative of eight mice.

for ^{51}Cr -release assays, we evaluated function over a broad range of E:T ratios from CTLs generated from four individual mice. The luminescence from the GLS.DEVD biosensor and the percentage of ^{51}Cr release increased proportionally with increasing E:T ratio (Fig. 3A, 3B). When we plotted percentage of total cell death in a 4-h ^{51}Cr -release assay against the 30-min luminescent signal in biosensor assay, we demonstrated remarkable correlation between the two assays with $R^2 \geq 0.95$ (Fig. 3C) and comparable sensitivities when titrating to low E:T ratios. Similar correlations were noted between the GLS.IETD signal and ^{51}Cr assay (data not shown). Thus, when evaluating cytotoxic function in mice with intact perforin and granzyme B pathways, the 30-min luminescent signal from the GLS.DEVD and GLS.IETD assays were equivalent to the gold standard cytolysis assay.

Target cell caspase 3/7 signal induced by CTLs is abrupt and transient

Whether using ex vivo CTLs or in vitro-derived CTLs, the area under the curve (luminescence versus time) was distinct when comparing the target cells expressing GLS.DEVD versus the granzyme B cleavable biosensors (Figs. 1–3). The luminescence from GLS.DEVD was detectable as early as 3 min following CTL/target coincubation, rising sharply until a brief plateau and/or decline was reached at ~30–60 min, whereas the GLS.IETD signal rose more slowly and continued to rise during the first 90 min. After 60 min, the fold activation of both biosensors was similar (Fig. 4D). We focused on the early activation signal of the GLS.DEVD biosensor, evaluating for the impact of effector number to determine whether enhanced granzyme B delivery impacted the rate of rise. As shown in Fig. 4A and 4B, the rate of luminescent activation was reduced at lower E:T ratios, although the plateau still occurred at the same time. When we evaluated the earliest time period for induction (from 10 to 30 min, Fig. 4C) we noted a range of GLS.DEVD activation from 7- to 70-fold induction (signal mediated by CTLs/signal from target alone at the same time) during 20 min, dependent on the E:T ratio.

The rapid increase of the GLS.DEVD signal within 3–10 min of coincubation suggested that caspase 3/7 activation was more rapid than previously recognized following CTL-mediated granzyme B delivery. To compare the caspase 3/7 activation mediated by murine CTLs to caspase activation induced by another method, we exposed the EL4 target cells to drugs known to induce apoptosis, such as etoposide (Supplemental Fig. 4) or staurosporine (not shown). Etoposide-mediated caspase 3/7 activation was readily detectable in a kinetic assay, with induction of a dose-dependent signal at 200 min following etoposide exposure. The kinetic induction of apoptosis in EL4 cells initiated by etoposide was delayed and gradual by comparison with that induced by CTLs (8-fold induction during 240 min for etoposide compared with 5- to 70-fold induction during 20 min for CTLs). Additionally, the GLS.DEVD signal rose steadily from 200 to 440 min without decline, when apoptosis was induced by etoposide. During the staurosporine induction, no signal was detectable in the 4-h window of measurement, although the signal was induced after overnight incubation (not shown).

The termination of the GLS.DEVD induction at 30–60 min was unexpected. This was visualized at all E:T ratios and is associated with a steep decline in signal at 30 min at the highest E:T ratio. A similar decline in signal was not observed for the GLS.IETD signal or other granzyme B-activated biosensors; however, at higher effector concentrations, there is a subtle and transient plateau of GLS.IETD at 40–45 min followed by a continuous rise (Fig. 4B, E:T ratios of 12:1 and 6:1) in luciferase. We do not know the precise mechanism for termination of the GLS.DEVD signal,

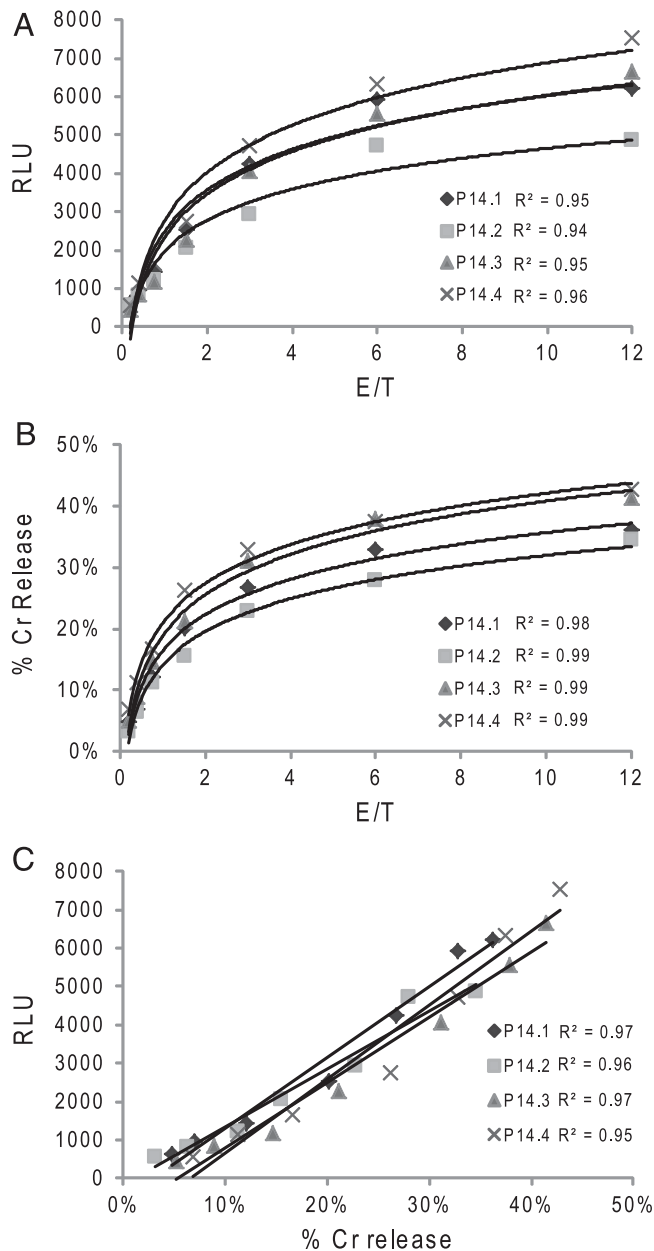


FIGURE 3. Biosensor signal correlates with gold standard ^{51}Cr -release assay. In vitro CTLs were generated by Ag stimulation of splenocytes from four individual P14 TCR transgenic mice. CTLs were either coincubated with biosensor-expressing cloned EL4 cells or ^{51}Cr -loaded EL4 target cells at E:T ratios ranging from 12:1 to 0.19:1. ^{51}Cr release was measured after 4 h. (A) RLU at 30 min of the luminescence assay for all four mice with titration of E:T ratio. (B) ^{51}Cr release percentage for all four mice with titration of E:T ratio. Best fit curves were logarithmic for (A) and (B). (C) Correlation between biosensor assay and ^{51}Cr assay. Data are representative of three independent experiments.

but several factors may contribute such as inactivation or degradation of active caspases, loss of cytosolic ATP (luciferase is ATP-dependent), or degradation of the cleaved luciferase (modeled in Fig. 4E). A similar phenomenon was reported previously in two unrelated cell lines expressing GLS.DEVD where luminescence was rapidly triggered by TRAIL within 120 min, followed by a decline in relative light units (RLU) (20).

We tested the dependence of the luciferase signal on caspase function and noted that addition of a pan-caspase inhibitor led to immediate drop in signal only within the first 30 min (addition at

time 0 and 30 min shown in Fig. 4F). The remaining decay in the RLU signal was largely caspase-independent and therefore likely related to dissociation of preactivated luciferase and/or loss of cellular ATP. The decline in signal was not due to a unique feature of cloned cell lines, as the data in Figs. 1D and 4F were obtained from polyclonal EL4 cells expressing GLS.DEVD. Thus, the kinetics for perforin-mediated, granzyme-activated caspase 3/7 is distinct, with rapid activation, followed by a plateau and/or decline of the early peak in the caspase 3/7-induced biosensor signal.

Distinguishing two independent mechanisms for CTL killing

CTL killing is mediated largely by two different pathways, the secretory granule (perforin/granzyme B) and the death receptor (Fas/FasL) pathways. Both mechanisms of cytotoxicity lead to caspase 3 activation, although the timing of the death receptor pathway is reported to be delayed and secondary to caspase 8 activation rather than granzyme B cleavage (35–37). Owing to the rapid kinetics of the luciferase induction noted from either biosensor, we predicted that the perforin-mediated pathway would be the sole determinate of GLS.DEVD and GLS.IETD biosensor activation in the first 90 min, whereas FasL-mediated killing would be detectable if the assay were extended beyond this early time point. We tested this by generating ex vivo CTLs from perforin-deficient and granzyme B-deficient mice. Coincubation of ex vivo CTLs from either immunodeficient strain with gp33-pulsed EL4 expressing GLS.DEVD or GLS.IETD led to luciferase induction only after the first 90–120 min (Fig. 5A, GLS.DEVD; GLS.IETD not shown).

CTLs from perforin-deficient mice triggered a delayed GLS.DEVD and GLS.IETD signal that continued to rise out to 4 h. To determine whether the delayed biosensor signal in GLS.DEVD and GLS.IETD was due to Fas/FasL-mediated activation, we tested CTL function in the presence of an anti-FasL Ab in perforin-sufficient and -deficient CTLs (P14 crossed to perforin-deficient mice). This was tested for both ex vivo CTLs (not shown) and in vitro-derived CTLs as shown in Fig. 5B and 5C. In vitro-derived CTLs were obtained from Ag-stimulated, IL-2-augmented splenocyte cultures from transgenic P14 mice. For both biosensors, at an E:T ratio of 1.5:1, the delayed biosensor signal from perforin-deficient CTLs was completely blocked in the presence of anti-FasL Abs. A portion of the decaying signal (starting at 90–120 min) from wild-type CTLs was also blocked. The biosensor activation from P14 CTLs was clearly perforin-dependent in the first 90 min, with a delayed signal occurring from Fas/FasL signaling. Thus, the biosensors detect both the rapid, perforin-dependent granzyme B induced caspase activation as well as the delayed, Fas/FasL-mediated caspase 3/7 (GLS.DEVD) or caspase 8 (GLS.IETD) activation. We also tested for delayed FasL/Fas activation in EL4 cells expressing GLS.IEAD and GLS.VGPD but did not observe a late signal. This suggests that these two biosensors are granzyme B specific and therefore unable to detect other caspase activity in apoptosis cells, although we cannot exclude the possibility of a low detection sensitivity of the biosensors.

The rate of activation of the GLS.DEVD signal by the FasL/Fas pathway in murine CTLs was slow in comparison with the induction mediated by the perforin/granzyme B-mediated pathway, whereas the rate of induction of GLS.IETD was similar for both pathways and the maximum signal was the same. The maximum signal obtained by the GLS.DEVD induced by either pathway was the same but took 2 h when mediated by Fas versus 30 min when induced by granzyme B. To our knowledge, these studies define for the first time the exact kinetics of FasL/Fas-induced caspase activation, confirm the absolute dependence of early caspase 3/7 activation on granzyme B delivery, and demonstrate the acceler-

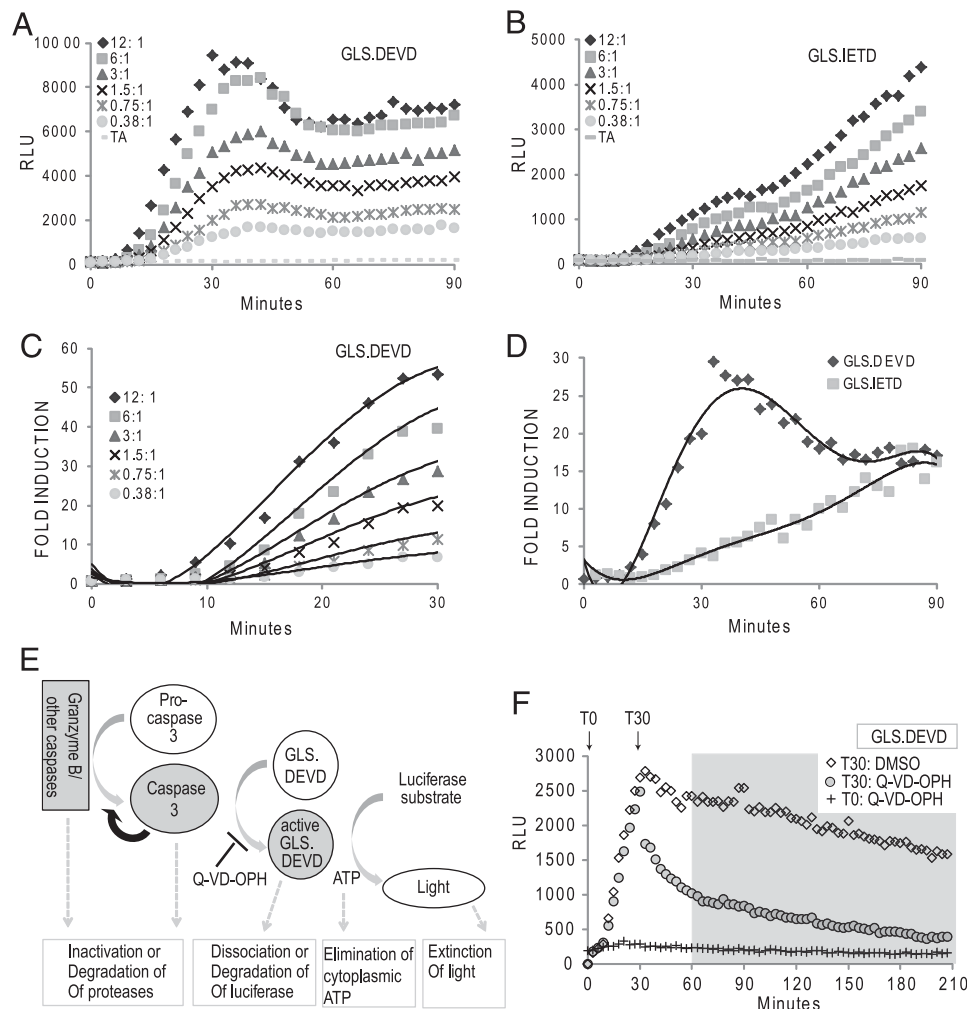


FIGURE 4. Caspase 3/7 activation of biosensor is rapid and transient from CTL induction. CTLs derived in vitro from a representative P14 mouse were coincubated with substrate-loaded target EL4 expressing GLS.DEVD (**A**) or GLS.IETD (**B**) as described in Fig. 3. (**C** and **D**) Fold induction of GLS.DEVD and GLS.IETD. RLU were graphed as a fold induction (signal generated with CTL/signal generated by target alone) at each time point up to 90 min. (**C**) The signal limited to the first 30 min of the GLS.DEVD assay is shown in higher detail to demonstrate the dependence of the fold induction on the E:T ratio. (**D**) Comparison of GLS.DEVD and GLS.IETD signals for the first 90 min illustrates the distinct kinetics of caspase 3/7 and granzyme B/caspase 8 activation. Data are representative of three independent experiments. (**E**) Model to show the potential mechanisms for acquisition and loss of GLS.DEVD signal. (**F**) Caspase dependency of GLS.DEVD activity. Using the same assay as described in (**A**) and (**B**), CTLs were coincubated with peptide-loaded EL4 cells and luminescence was monitored from GLS.DEVD out to 4 h. A pan-caspase inhibitor, Q-VD-OPH, was added at the start of the assay, T0, or at 30 min, T30. There was complete inhibition of GLS.DEVD when Q-VD-OPH was added at T0. Following addition at T30, there was an immediate dramatic loss of signal to 60 min compared with cells exposed to DMSO, illustrating the dependence of the biosensor on caspase signaling from 0 to 60 min. There is a caspase-independent loss of activity from 60 to 240 min (delineated by gray box) with nearly identical degradation in the presence or absence of caspase inhibition. (**A**–**D**) Experiments used cloned EL4 cells expressing GLS.IETD or GLS.DEVD. (**F**) Experiments used polyclonal, GFP-sorted EL4 cells expressing GLS.DEVD.

ated rate of caspase 3/7 induction by the secretory granule pathway. Finally, the lack of early signal in the GLS.IETD (not shown) and GLS.DEVD (Fig. 5A) sensors following induction of apoptosis by granzyme B-deficient CTLs demonstrates the absolute dependence of these biosensors on granzyme B delivery as predicted. We conclude that the GLS.DEVD and GLS.IETD biosensors may be used to study both perforin/granzyme-mediated apoptosis as well as late phase, death receptor-induced apoptosis.

To generalize our findings obtained using murine CTLs, we tested the capacity of the GLS.DEVD biosensor to detect caspase activation from human CTLs. We tested both primary T lymphoblast cultures obtained following mitogen stimulation (not shown) and transformed, stable human T cell cultures obtained following HVS transformation. The advantage of the latter is the stability of these cultures over time and tolerance for freeze/thaw. They have previously been shown to exhibit strong cytotoxicity

following redirected killing, a method that relies on activation of T cells via Ab stimulation of CD3, rather than an Ag-specific T cell (38). We derived HVS CTLs from both a healthy control and a perforin-deficient patient who presented with hemophagocytic lymphohistiocytosis due to biallelic, truncating perforin mutations (50delT) and absent NK function.

For assays of human CTL function by redirected killing, the GLS.DEVD biosensor was cloned by limiting dilution into P815, a murine tumor line that binds murine Ig via Fc receptor. Upon coincubation of P815 cells with anti-CD3 Ab and HVS T cells from a healthy donor at an E:T ratio of 1, we saw activation of the biosensor within 10 min, plateauing at 90 min, followed by a gradual, persistent decline in luciferase signal (Fig. 6A). The luciferase signal was inhibited by concanamycin A, a compound previously shown to ameliorate perforin-mediated cytotoxic function (39), whereas brefeldin A, which inhibits FasL upregulation on T cells, had no impact.

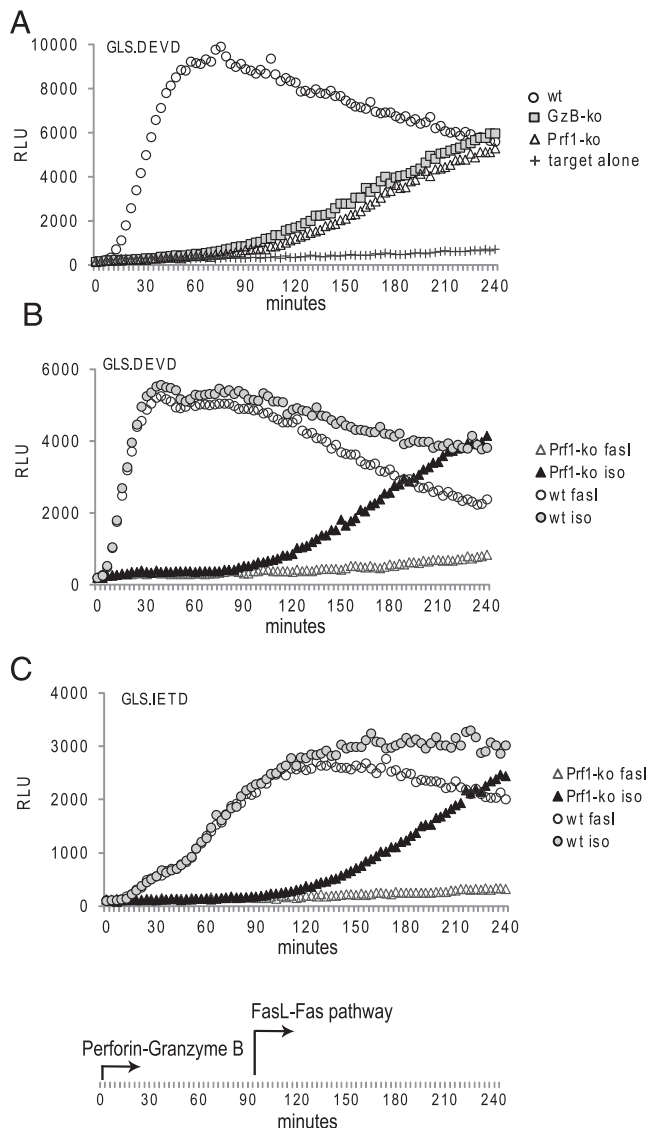


FIGURE 5. Caspase 3/7 activation mediated by perforin-deficient murine CTLs is detectable following late phase Fas-mediated induction. **(A)** Ex vivo CTLs were generated in wild-type (WT) C57BL/6, perforin-deficient (Prf1 ko), and granzyme B deficient knockout (GzB ko) mice infected with LCMV for 7 d. Splenocyte/EL4 target ratio of 12:1 is shown for a representative mouse (four mice per group tested in three independent assays). Evaluation of RLU out to 240 min reveals a late activation of the GLS.DEVD (A) and GLS.IETD biosensors (not shown) in mice lacking perforin-mediated granzyme B delivery. **(B and C)** P14 in vitro CTLs from WT and perforin-deficient mice were also tested as effectors. Addition of anti-FasL Ab to the reaction prevented the delayed rise in GLS.DEVD (B) and GLS.IETD (C) signal in perforin-deficient mice, illustrating the delayed onset of the death receptor pathway (≥ 90 min). Target cells were polyclonal EL4, sorted by GFP following retroviral transduction of luciferase biosensors.

We then tested the function of perforin-deficient HVS T cells and noted that these cells activated the GLS.DEVD biosensor only after a 90-min delay (Fig. 6B). This rise in signal was blocked using anti-FasL Ab (Fig. 6B) or brefeldin A (not shown). The rate of induction of the Fas-mediated signal from the perforin-deficient T cells was slower than the induction of the granzyme-mediated signal from the perforin-sufficient cell line, although this difference was not as pronounced as the differences seen in murine CTLs. Remarkably, the timing of induction following Fas activation was the same as that seen using perforin-deficient murine CTLs, with a gradual rise in signal occurring only after a 90- to 120-min delay.

This technology allows the capacity to precisely time the induction of delayed Fas/FasL-mediated caspase activation following the immediate induction achieved by perforin-mediated granzyme B delivery. Additionally, the method allows comparison of the rate of induction of caspase induction by the two different pathways of cytotoxicity and signaling via two different immune synapses (MHC-peptide versus anti-CD3 Ab).

Discussion

We have shown that circularly permuted luciferase proteins may be engineered as protease biosensors to measure CTL-mediated granzyme B delivery and subsequent caspase activation in live target cells. This novel luminescence-based CTL assay will be an invaluable tool for mechanistic assessment of CTL cytotoxicity. Utilizing a combination of caspase 3/7 and granzyme B-cleavable biosensors, we have shown that the caspase 3/7 signal induced by CTL is extremely rapid and transient, contrasting to the signal obtained utilizing granzyme B-cleavable biosensors.

Previous studies revealed the absolute dependence of murine granzyme B to induce caspase 3 activation following recognition of target cells (12, 40, 41) by CTLs, and they demonstrated caspase 3 activation in target cells by flow cytometry following CTL contact (10–12). Caspase 3 activation is also noted in activated T cells (42–46), limiting the utility of caspase 3 activation as a measure of CTL function using freely diffusible fluorescent substrates in a 96-well format. As such, to our knowledge this luciferase biosensor assay is the first cell-based method that detects CTL activation of caspase 3/7 in target cells in a microplate format.

We engineered a novel biosensor with a cleavage site for both granzyme B and caspase 8 (IETD). In the absence of granzyme B, there was no activation GLS.DEVD or GLS.IETD within 90 min, confirming the absolute dependence on granzyme B for caspase 3/7 and caspase 8 activation induced by secretory granule-mediated killing. Additional protease recognition sites specific for alternative granzymes may also be introduced in future studies to investigate their role in apoptosis in the presence or absence of granzyme B. Two such sites, IEAD and VGPD, were screened in the current study, but not pursued due to their suboptimal signal-to-noise ratio in EL4 target cells.

The luciferase biosensor approach provides several advantages over conventional radioactive and flow-based CTL cytotoxic assays: 1) a homogeneous, microplate format without radioactivity; 2) real-time assessment of early events of target cell apoptosis in live cells; and (3) amenability to a high-throughput format. The main limitation of the assay relates to standardization of the assay results. Unlike a ^{51}Cr -release or flow-based assay, there is no “maximum” signal. One approach is to perform the assay using a range of E:T ratios to find the maximum signal per assay. Indeed, we have found that beyond CTL/target ratios of 6:1 to 12:1 there is no increase in luminescent signal (not shown). An ideal approach would induce apoptosis in target cells by a cell-free method (such as shown with etoposide) to provide a CTL-independent, maximum signal in parallel with the CTL cytotoxicity assay. We are currently investigating a compound that may induce apoptosis within minutes, analogous to the CTL-induced pathway. Alternative standardization may use measures of fold induction compared with the basal signal in the negative control as shown for Fig. 4 and Supplemental Fig. 4.

We have measured caspase 3 activation in EL4 cells using other methodologies to evaluate the phenomenon of rapid caspase 3 activation mediated by murine CTLs. Unfortunately, measuring active caspase 3 by fluorogenic substrate or Ab recognition of the active form was not sensitive at early time points to show induction of caspase 3 in EL4 cells within the first 30 min of CTL coin-

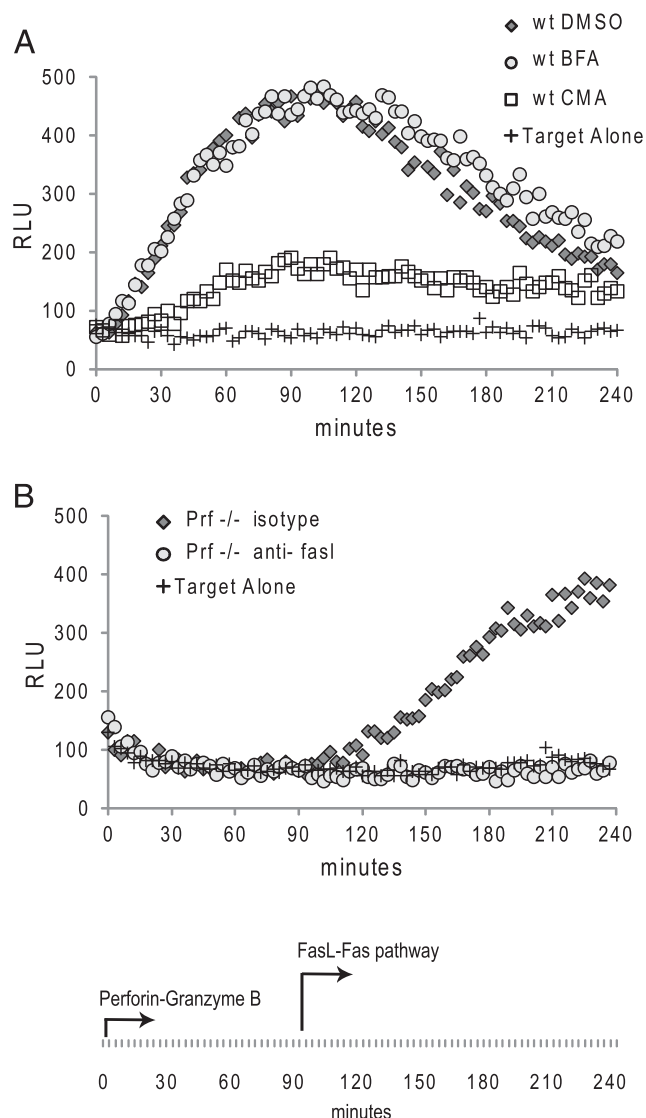


FIGURE 6. Redirected killing: human CTLs use secretory granule and Fas-mediated pathways to activate caspase 3/7 in target cells conjugated by anti-CD3. **(A)** HVS-transformed human CTLs were coincubated at 1:1 with GLS.DEVD-expressing cloned P815 cells preincubated with anti-CD3 Ab. P815 cells without added effectors are shown as a negative control. Evaluation of RLU out to 240 min reveals early activation of GLS.DEVD in P815 incubated with wild type (wt) CTLs contrasting with **(B)** late activation following coincubation with perforin-deficient CTLs (*Prf*^{-/-}). The early activation in the wt cell line was prevented by the addition of concanamycin A (CMA) but not brefeldin (BFA), demonstrating the dependence on the secretory granule pathway. Addition of anti-FasL Ab to the *Prf*^{-/-} CTLs prevented the delayed rise in GLS.DEVD (B), confirming the role of the delayed onset death receptor pathway. Shown is a representative figure from three independent experiments utilizing these two HVS lines.

cubation (data not shown). This is likely because the fluorogenic substrates have substantial background in EL4 cells (12). Evaluating caspase 3 cleavage in EL4 target cells by Western blot following coincubation with CTLs was unreliable for two reasons: 1) CTLs have significant amounts of caspase 3, and 2) cleavage of caspase 3 by granzyme B occurs during the lysate preparation. The latter could be overcome with the use of a granzyme-specific inhibitor, but none is currently available for murine granzyme B.

The luminescent assay permits serial measurements of caspase activation during the entire time course of the assay. Real-time evaluation of kinetics is straightforward, using a heated platform

and buffered media to preserve CTL function outside of the tissue culture incubator. Our data demonstrate that delivery of granzyme B to the target cell by the effector CTLs is extremely rapid, consistent with previous studies using ⁵¹Cr-release assays or direct visualization (5, 6, 47). Therefore, both biosensors detect the earliest events of cytotoxicity. Indeed, the signal at 30, 60, and 90 min correlates well with the eventual cytolysis of target cells at 4 h (Fig. 3 and data not shown).

The kinetics for GLS.DEVD induction in target cells were slightly different when comparing in vitro-derived and ex vivo murine CTLs, highlighting the capacity of the biosensors to distinguish CTL function. The in vitro-derived CTLs induced a caspase-mediated signal that peaked at 30–40 min whereas the ex vivo CTLs induced a peak signal at 60 min. The difference in the kinetics was not explained by the lower E:T ratio for the ex vivo CTLs, as the cultured CTLs behaved similarly at low E:T ratios (although the peak was less distinct). However, we did not try to dilute the target cells with an irrelevant cell to test whether the delayed timing was related to a delay in cell contact for the ex vivo CTLs, which were diluted in the assay by nonresponding splenocytes. Future studies may be directed to understand whether the differences are related to differences in cellular adhesion or perforin/granzyme B levels.

Comparison of GLS.DEVD versus GLS.IETD signals in the earliest time points following in vitro-derived CTL induction also revealed distinct kinetics, including the rate of luminescence activation and the rate of luminescence decline. Although both the GLS.DEVD and GLS.IETD sensors were activated upon contact of effector CTLs and target cells, the overall signal induction was faster and 2- to 5-fold higher for GLS.DEVD, which likely reflects the autoamplification property of caspase 3 following granzyme B cleavage (48, 49). Following contact with cultured P14 CTLs, the GLS.DEVD signal declined rapidly beyond 30 min, whereas the GLS.IETD signal plateaued briefly (30–45 min) and then continued to rise during the entire 90 min. Previous studies have predicted CTL/target cycles of contact lasting ~20–30 min (50–52). The brief plateau in the IETD signal at 30 min may thus reflect the beginning of the second cycle or a pause between cell contact. The subsequent decline noted in the EL4 cells expressing the GLS.DEVD suggests that the initial caspase 3/7 activation signal may be turned off in a fraction of the earliest responding cells at 30 min. This may be related to inactivation of caspase 3/7 by endogenous inhibitors (53, 54), dissociation of the luciferase, or to rapidly declining ATP levels in these early responding cells. Although we would expect the GLS.IETD signal to similarly decline if related to cell death/ATP loss, the loss of signal may be too low to detect in this biosensor where there is no amplification of the signal from caspases other than granzyme B. Further studies are needed to investigate this phenomenon.

The kinetics of caspase 3/7 activation following the Fas/FasL-mediated apoptosis pathway was also distinct from that induced by the secretory granule pathway. Our data in perforin-deficient murine and human CTLs suggest that in the first 90 min there is no detection of Fas/FasL-mediated activation of caspase 3/7 or caspase 8 (Figs. 5, 6). Because CTLs from perforin-deficient animals are unable to kill target cells as measured by a ⁵¹Cr-release assay (data not shown), our data confirm that perforin deficiency is not compensated for by Fas/FasL activation in the first 90 min of CTL/target contact. Late activation of the Fas/FasL path was clearly visualized in perforin- and/or granzyme-deficient CTLs. The delay in initiation of the death receptor pathway and the gradual induction of caspase activation observed may be interrogated further with this novel system of measuring caspase activation.

In summary, using a novel, luminescence-based assay for measuring CTL function we have detected granzyme B-mediated apoptosis within live target cells in real time. This approach is an important technological advance, because measurement of target cell protease activity in a typical CTL assay (with two independent cell populations present) has only been feasible by flow cytometry. The rate of activation was modulated by the number of CTLs interacting with each target cell and the strength of TCR signaling. We predict that the luciferase biosensor assay will rapidly become a widely used method for measuring cytotoxic function in CTLs. Our initial studies with murine NK cells were somewhat disappointing, but may require use of a different target cell line. Indeed, we have already adapted the real-time detection assay to human NK targets (J. Li and K.A. Risma, manuscript in preparation). We also predict that the microplate assay will be readily adaptable to a high-throughput screening format, allowing transformative studies to address genetic and pharmacologic modifiers of cytotoxic function.

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Disclosures

B.F.B. and B.L.B. are employees of Promega Corp. and, as such, have conflicts of interest. The other authors have no financial conflicts of interest.

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