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Rapid and Unidirectional Perforin Pore Delivery at the Cytotoxic Immune Synapse

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The effective engagement of cytotoxic lymphocytes (CLs) with their target cells is essential for the removal of virus-infected and malignant cells from the body. The spatiotemporal properties that define CL engagement and killing of target cells remain largely uncharacterized due to a lack of biological reporters. We have used a novel live cell microscopy technique to visualize the engagement of primary human and mouse CL with their targets and the subsequent delivery of the lethal hit. Extensive quantitative real-time analysis of individual effector–target cell conjugates demonstrated that a single effector calcium flux event was sufficient for the degranulation of human CLs, resulting in the breach of the target cell membrane by perforin within 65–100 s. In contrast, mouse CLs demonstrated distinct calcium signaling profiles leading to degranulation: whereas mouse NKs required a single calcium flux event, CD8+ T cells typically required several calcium flux events before perforin delivery. Irrespective of their signaling profile, every target cell that was damaged by perforin died by apoptosis. To our knowledge, we demonstrate for the first time that perforin pore delivery is unidirectional, occurring exclusively on the target cell membrane, but sparing the killer cell. Despite this, the CTL membrane was not intrinsically perforin resistant, as intact CTLs presented as targets to effector CTLs were capable of being killed by perforin-dependent mechanisms. Our results highlight the remarkable efficiency and specificity of perforin pore delivery by CLs. *The Journal of Immunology, 2013, 191: 000–000.

Cytotoxic lymphocytes (CLs) identify pathogen-infected and transformed target cells and eliminate them primarily through granule-mediated apoptosis (1). Upon conjugation with their targets and CL signaling, receptors on the CL cell surface undergo rapid reorganization into a tight immunological synapse (IS) (2). IS formation triggers exocytosis of lytic granules containing the effector molecules perforin and granzymes, into the synaptic cleft, in a process known as degranulation (3, 4). Perforin forms transmembrane pores in the target cell, allowing direct diffusion of extracellular milieu, including granzymes into the target cell cytosol (5), where they activate apoptosis pathways through proteolytic substrate processing (6). Importantly, perforin pore formation on the target cell plasma membrane represents the most proximal and essential step in the killing of the target cell.

Regulated Ca2+ flux following receptor activation is essential for CL degranulation and target cell killing and represents an early CL signaling event (7). Humans deficient in the Ca2+ ion channel protein ORAI-1 have reduced CL degranulation and killing function and present with a severe immunodeficiency (8). Calcium signaling most likely impinges on the granule trafficking pathways through regulation of Ca2+-responsive proteins, including myosin (9), synaptotagmin (10), Munc13 (11, 12), and Doc2 (13), to facilitate the rapid movement and fusion of secretory granules with the plasma membrane. However, it is unclear how Ca2+ signaling dictates degranulation events in the CLs, whether there is a rapid response to elevated intracellular Ca2+, and also whether degranulation requires a single Ca2+ flux or multiple bursts.

Many of the processes governing effector function are conserved between CTLs and NK cells, but these cells have also evolved specialized properties to meet the unique niches of the innate and adaptive immune responses. Importantly, the mode of target cell recognition, engagement, and intracellular signaling events largely underpin their specificity (14). Recent in vivo imaging studies showed that NK cells tend to make transient contacts with their tumor targets, whereas CTLs form longer, more stable contacts (15). The authors suggested these differences in contact time could be explained by differences in calcium responses, with NK cells showing weak calcium influxes, whereas CTLs showed high, sustained calcium influxes. In these experiments, flow cytometry was used to estimate changes in calcium within conjugated effector cells, but the temporal relationship to degranulation was not examined.

The transient nature of effector-target interactions presents challenges for studies aimed at dissecting the cytotoxic immune synapse. Fixed cell sampling of CL-target conjugates offers little insight into the dynamic nature of synapse formation, and the lack of a functional reporter for lethal hit (perforin) delivery precludes...
the objective analysis of time-lapse images. Therefore, previously it has not been technically possible to investigate the dynamics of NK- and CTL-target cell engagement in detail. We have very recently developed a new tool to measure the effects of CL degranulation in real time (5). Using this method, we now for the first time, to our knowledge, directly visualize permeabilization of the target cell membrane by secreted CL perforin, from human and mouse NK cells and CTLs, within bona fide immunological synapses. This technique is simple, direct, and readily adaptable for various effector-target cell combinations. In this study, we have coupled this technique with calcium signaling measurements in the killer cell, to characterize the spatiotemporal properties of CL target engagement and subsequent delivery of the lethal hit.

Materials and Methods

Reagents

The calcium indicator, Fluo-4 AM, and its carrier pluronic acid F-127 were from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell culture

HeLa human cervical carcinoma cells and MC57 fibrosarcoma cells were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS and 2 mM l-glutamine. BOLETH (HC10329) human B cell targets were maintained in RPMI 1640 medium (Invitrogen) supplemented with 5% (v/v) FCS, 2 mM l-glutamine, and 100 μM nonessential amino acids.

Purification of human and mouse NK cells

Primary human or mouse NK cells were isolated from peripheral blood of healthy donors or from mouse spleen, respectively, by negative selection using the MACS NK isolation kit and autoMACs separator (MACS; Miltenyi Biotec). Isolated human NK cells were maintained at a density of 1 × 10^6 cells/ml in RPMI 1640 NK medium (10% [v/v] heat-inactivated FCS and 2 mM glutamine, 5% [v/v] human serum, 2 mM l-glutamine, 100 μM nonessential amino acids, and 100 U/ml rIL-2).

Purification of mouse CTLs

Primary CTLs were isolated from the spleens of C57BL/6-OT-I mice and activated with OVA257 peptide, as described previously (16). Cells were Ficoll purified on day 4 and used for experimentation on day 6 or 7.

Live cell confocal microscopy

Target cells were prepared for live cell imaging by seeding 3 × 10^4 HeLa or MC57 cells into each well of an 8-well chamber slide (ibidi, Munich, Germany) and incubating overnight at 37°C/10% CO2. For CD8+ T cell experiments, MC57 and Prf1−/− CTLs or BOLETH target cells were pulsed with 1 μM OVA257 or B2QK-86 peptide, respectively. Suspension target cells were adhered to chamber slides 15 min prior to imaging, by incubating in serum-free media at 37°C. A total of 2 × 10^5 Fluo-4-labeled (labeled for 20 min with 1 μM Fluo-4 and 0.02% [v/v] Pluronic F-127 carrier at 37°C/10% CO2) NK cells or CTLs was added to 8-well chamber slides containing adherent targets, in media containing 100 μM PI. Chamber slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C, and constant CO2 concentrations (5 or 7%)

were infused using a gas incubation system with active gas mixer (“The Brick”; ibidi). Optical sections were acquired through sequential scans of Fluo-4 (excitation 488 nm; 5-μm pinhole), propidium iodide (PI) (excitation 561 nm; 1.38-μm pinhole), or Brightfield/DIC on a TCS SP5 confocal microscope (Leica Microsystems, Deerfield, IL) using a 40× (NA 0.85) air objective and Leica LAS AF software. Images were acquired at ~6–7 frames/min.

Image analysis

Image analysis was performed using Leica LAS AF Lite software or MetaMorph Imaging Series 7 software (Universal Imaging, Downingtown, PA).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 software.

Ethics

Studies involving human tissue were approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (approval number 12/73) and St. Vincent’s Hospital Human Research Ethics Committee (approval number 135/08).

Results

To investigate the interactions of CLs and their cognate targets leading to target cell death, we have developed an approach that for the first time, to our knowledge, enables the precise detection of target cell membrane damage by perforin in real time, signifying delivery of the lethal hit (5). The detection of membrane damage by perforin is based on the diffusion of large (8 Å) and membrane-impermeable, extracellular PI through perforin pores (150 Å) and the formation of fluorescent cytosolic PI/RNA complexes. The highly fluorescent and stable PI/RNA complex enables the continuous tracking of targets over time (5). We applied this technology to investigate the spatiotemporal behavior of CL target cell engagement and delivery of the lethal hit.

We first examined the temporal relationship between Ca2+ signaling and delivery of the lethal hit by human and mouse CTL and NK cells. Using the calcium fluorophore Fluo-4 to label CL populations, we found that Ca2+ flux, a proximal signaling event leading to degranulation, occurred very rapidly following conjugation of human primary NKs with their targets (Fig. 1A, Supplementary Video 1). During this initial contact, the NK cell cytosolic Ca2+ levels rapidly increased, and breach of the target cell membrane by perforin occurred soon after the peak of the Ca2+ response (Fig. 1A, 1B); this was indicated by the focal uptake of extracellular PI specifically at the IS (Fig. 1A; 110 s). Fluo-4 fluorescence and cytosolic Ca2+ were restored toward baseline (starting fluorescence levels) following delivery of the lethal hit. We made similar observations with human CTLs: permeabilization of the target cells occurred soon after the peak in CTL cytosolic Ca2+ (Fig. 1C, 1D, Supplementary Video 2).

We next measured the interval between the initial rise in NK Ca2+ (as detected by Fluo-4) and perforin delivery to the target (as indicated by PI/RNA fluorescence), using NK cells from five healthy human donors. The interval was uniformly rapid and consistent among the donors (67 ± 8 s, mean ± SD; Fig. 1E), despite their genetic diversity. These results were comparable to human CTL clones, which also rapidly and consistently delivered perforin after Ca2+ flux (99 ± 11 s, mean ± SD; Fig. 1F).

To assess the conservation of calcium-regulated degranulation, we next examined immune synapses of mouse NKs and CTLs with their respective targets. Similar to human CLs, a single influx of Ca2+ into mouse NKs was required for the delivery of perforin to the target cell (Fig. 2A, 2B, Supplementary Video 3). However, in contrast to human NK cells, the lethal hit delivery was significantly delayed (160 ± 21 s, mean ± SD; Fig. 2F). These apparent
interspecies kinetic differences may reflect a number of parameters, including signal strength, differences in susceptibility between the various target cells, and activation status of human and mouse cells. Mouse NK cells, for example, were isolated from spleen and activated in vitro for several days, whereas naive human NK cells were isolated from peripheral blood.

FIGURE 1. A single global increase in intracellular calcium precedes delivery of the lethal hit by human CLs. (A) Time-lapse microscopy of primary human NK cells with HeLa target cells in the presence of 100 μM PI: images were acquired every 10 s and show Fluo-4 (green)/PI (red)/Brightfield overlay. Images depict conjugation of the NK with the HeLa target (20 s), elevation in NK intracellular calcium (initiated at 30 s), followed by diffusion of PI into the target cell cytosol (initiated at 110 s). Scale bar, 10 μm; images are representative of 10–15 cells from each of five independent experiments (using five different human donors). See also Supplemental Video 1. (B) Quantitation of the Fluo-4 fluorescence change over time in primary human NK cells relative to uptake of PI by the target cells; images were acquired every 10 s. Data represent the fold change (F/F₀) in Fluo-4 and PI fluorescence over time. Arrows indicate the time of cytosolic calcium increase in the effector and the time of PI uptake by the target. (C) Time-lapse microscopy of primary human CD8⁺ T cell clones with B cell target cells in the presence of 100 μM PI: images were acquired every 10 s and show Fluo-4 (green)/PI (red)/Brightfield overlay. Images depict conjugation of the CD8⁺ T cell with the B cell target and elevation in T cell intracellular calcium (initiated at 70 s), followed by diffusion of PI into the target cell cytosol (initiated at 150 s). Scale bar, 10 μm; images are representative of 12–13 cells from each of three independent human clone experiments. See also Supplemental Video 2. (D) Quantitation of the Fluo-4 fluorescence change over time in primary human CD8⁺ T cell relative to uptake of PI by the target cells; images were acquired every 10 s. Data represent the fold change (F/F₀) in Fluo-4 and PI fluorescence over time. Arrows indicate the time of cytosolic calcium increase in the effector and the time of PI uptake by the target. (E and F) Rapid engagement of CLs with their target cells ensures efficient delivery of lytic granules; the timing between the initial calcium flux by effector cells and the uptake of PI by target cells. (E) Primary human NK effectors and HeLa targets; data represent the mean ± SD of 10 cells from each of 5 independent human donors (n = 50). (F) Primary human CD8⁺ T cells and B cell targets; data represent mean ± SD of 12–13 cells from each of three independent human clones (n = 37).
We also assessed Ca\(^{2+}\) signaling and degranulation properties in OVA Ag-specific mouse CTL populations. Interestingly, in contrast to all other CL populations, mouse CTL conjugation with their targets was immediately followed by between one and seven fluctuations in cytosolic calcium (2.7 ± 1.7, mean ± SD) prior to permeabilization of the target (initiated at 340 s). Scale bar, 10 μm; images are representative of 12–17 cells from each of three independent mice. See also Supplemental Video 4. The interval between the first Ca\(^{2+}\) flux and delivery of the lethal hit was significantly longer than with other CLs, but was remarkably consistent for the CTLs of three different mice (26 ± 5 s, mean ± SD; Fig. 2G). Importantly, single Ca\(^{2+}\) flux events were observed with some mouse CTLs, indicating that Ca\(^{2+}\) prepriming of the exocytotic granule pathway (e.g., calcium-dependent cytoskeletal changes and/or granule recruitment) is not essential for their cytotoxic action.

Our analysis of NK-target interactions revealed that a small proportion of human NK cells delivered multiple hits to a single target cell (Fig. 2F). This phenomenon was not observed with mouse CTLs but was consistent with the findings in a previous study. The number of distinct calcium flux events in the human NK cells correlated with the number of multiple hits delivered, suggesting that the number of Ca\(^{2+}\) flux events may be a useful indicator of the cytotoxic potential of these cells.

**FIGURE 2.** Distinct calcium signaling profiles initiate degranulation and delivery of the lethal hit by mouse CLs. (A) Time-lapse microscopy of primary mouse NK cells with MC57 target cells in the presence of 100 μM PI: images were acquired every 10 s and show Fluo-4 (green)/PI (red)/DIC overlay. Images depict conjugation of the NK with the MC57 target and elevation in NK intracellular calcium (initiated at 50 s), followed by diffusion of PI into the target cell cytosol (initiated at 340 s). Scale bar, 10 μm; images are representative of 12–17 cells from each of three independent mice. See also Supplemental Video 3. (B) Quantitation of the Fluo-4 fluorescence change over time in primary mouse NK cells relative to uptake of PI by the target cells; images were acquired every 10 s. Data represent the fold change (F/F₀) in Fluo-4 and PI fluorescence over time. Arrows indicate the time of cytosolic calcium increase in the effector and the time of PI uptake by the target. (C) Time-lapse microscopy of primary mouse CD8\(^{+}\) T cells with MC57 target cells in the presence of 100 μM PI: images were acquired every 8.6 s and show Fluo-4 (green)/PI (red)/Brightfield overlay. Images depict conjugation of the CD8\(^{+}\) T cell with the MC57 target and sequential elevations in T cell intracellular calcium (initiated at 25 s: Calcium Flux 1 and at 77 s: Calcium Flux 2), followed by diffusion of PI into the target cell cytosol (initiated at 146 s). Scale bar, 10 μm; images are representative of 10–16 cells from each of three independent mice. See also Supplemental Video 4. (D) Quantitation of the Fluo-4 fluorescence change over time in primary mouse CD8\(^{+}\) T cells relative to uptake of PI by the target cells; images were acquired every 8.6 s. Data represent the fold change (F/F₀) in Fluo-4 and PI fluorescence over time. Arrows indicate the time of cytosolic calcium increase in the effector and the time of PI uptake by the target. (E) Measurement of the number of distinct calcium flux events in mouse CD8\(^{+}\) T cells following conjugation with their targets and prior to delivery of the lethal hit (as detected by PI uptake); data represent mean ± SD of 10–16 cells from each of three independent mice (n = 39). (F and G) The timing between the initial calcium flux by effector cells and the uptake of PI by target cells. (F) Primary mouse NK effectors and MC57 targets; data represent mean ± SD of 12–17 cells from each of three independent mice (n = 42). (G) Primary mouse CD8\(^{+}\) T cells and MC57 targets; data represent mean ± SD of 10–16 cells from each of three independent mice.
target cell (<10%), consistent with a recent study (19). By analyzing the uptake of PI by the target over time, we observed up to three successive pulses of cytosolic PI (hits 1, 2, and 3; Fig. 3A, corresponding Supplemental Videos 5, 6). In each case, PI uptake was transient, as demonstrated by the rapid increase and decrease of PI fluorescence at the focal point of the IS, indicating that the target cell repaired the damage between each successive hit (5). These data demonstrated that prolonged target cell engagement by NK cells could result in multiple degranulation events. Multiple hits to a single target were also detected for mouse NKs (Supplemental Fig. 1). The sequential delivery of hits by a single effector cell is unlikely to be necessary for death of a single target, because, irrespective of the magnitude of the hit (as measured by the change in PI fluorescence over time), every target cell damaged by perforin died by apoptosis. However, the sequential delivery of hits is likely to be important for their serial killing function. More than 90% of serial killing events that we recorded involved rapid sequential delivery of perforin to different, but closely adjacent target cells, whereas the killer cell was evidently simultaneously in contact with both targets (Fig. 3B, corresponding Supplemental Videos 7, 8).

How CLs can enforce multiple rounds of killing and remain protected from their toxic effector molecules has been a long-standing puzzle. It was previously proposed that neutralization of granzymes might confer protection, for instance by expression of high levels of serpins, inhibitors of serine protease activity, within CLs (20). However, we found that the uptake of PI was almost invariably unidirectional (Fig. 4A), indicating that perforin pores were not formed on the CL and therefore, secreted granzymes could not access the cytosol of the killer cell. In total, we have examined 413 functional CLs synapses that resulted in target cell death, yet only 2 CLs (<1%) took up PI, both of which died after hitting their target. These data directly and graphically demonstrate for the first time, to our knowledge, the specificity of perforin’s action on the target cell.

We next asked whether CLs have evolved a specialized mechanism that confers resistance to perforin. To test this possibility, Fluo-4–labeled wild-type CTLs from transgenic (OT-1) mice were

![FIGURE 3. Rapid sequential degranulation events ensure efficient serial killing by human CLs. (A) CLs deliver multiple hits to single target cells; monitoring the PI fluorescence change over time in a single HeLa target cell, following conjugation of a primary human NK cell. Images were acquired every 5.5 s. Data represent the fold change in PI fluorescence (F/F0) over time at a site proximal to the immune synapse; arrows demarcate delivery of three distinct lethal hits (hits 1, 2, and 3). See also Supplemental Video 5. (B) Rapid sequential delivery of lethal hits. Upper panel. Quantitating the PI fluorescence change over time from two target cells using a single NK effector; data represent the fluorescence change (F/F0) in HeLa targets (PI fluorescence); arrows indicate the time of PI uptake (Hit 1 and Hit 2) by the targets. The shaded bars represent the first and second degranulation events, respectively. Each degranulation event hits both target cells, with the magnitude of each hit determined by the migrating NK cells’ proximity to each individual target. Lower panel. Schematic representation of a single NK cell engaging with its targets, resulting in two rounds of degranulation, as measured by PI uptake in two target cells (red arrow indicating uptake of PI by Target Cell 1 and Target Cell 2). See also Supplemental Video 7.](http://www.jimmunol.org/Download/FIG3.jpg)
incubated with OVA peptide (SIINFEKL)-pulsed CTLs of Prf1−/− OT-1 syngeneic mice that had been generated in an identical fashion. We found that in 42 of 44 cell death events recorded (>95%), the CTL that inflected the killing remained resistant to PI uptake, whereas PI diffusion was readily observed in CTL targets, resulting in rapid apoptotic death (Fig. 4B, 4C, Supplemental Video 9). These data directly demonstrated that effector cells were refractory to the formation of the transmembrane perforin pore, and are consistent with the unidirectional fratricide-based killing observed previously by others (21). These results strongly argue that protection from perforin is not an intrinsic property of the CL membrane, but rather is acquired as a consequence of degranulation. It remains to be determined how degranulation confers this protection, whether through inhibition of perforin binding or perforin pore formation per se.

Discussion
The efficiency of CL serial killing is determined by the ability of CLs to rapidly form functional immune synapses with specific cell targets. Determining how and when these CL synapses form is critical for our understanding of CL biology. Our study defines the spatiotemporal properties governing engagement of CLs with their target and delivery of the lethal hit and further demonstrates the unidirectionality of perforin pore formation at the IS.

The slower degranulation kinetics and multiple calcium flux events of mouse CTLs compared with mouse NK cells are consistent with the longer-lived target contact times and sustained calcium signaling profiles observed previously with mouse CTLs (15). Interestingly, NK cells appear to degranulate more quickly upon target cell engagement, irrespective of species. Although such differences in degranulation kinetics and contact time may have important consequences on the capacity of CTLs and NK cells to engage in further killing events, we cannot exclude the possibility that distinct preparations of our CLs could influence the killing kinetics.

The sequential delivery of hits by NK cells is in agreement with a previous study (22), which reported that cytotoxic synapses could simultaneously form between a single CTL and more than one target. It was hypothesized that, in such circumstances, lytic granules may be directed to different plasma membrane contact points via separate signaling events. We propose that this cytotoxic mechanism also occurs in NK cells and is particularly deployed if both targets are located in the same direction as the NK’s polarized centrosome. Simultaneous or sequential engagement with adjacent targets would ensure rapid serial killing, as the centrosome can rapidly reorientate to an adjacent membrane (23). However, recent studies proposed that lytic granule secretion may not even require prior polarization of the centrosome (24). Our data describing the sequential delivery of lethal hits with adjacent targets are consistent with a recently published study that investigated the kinetics of serial killing using the NK cell line, NK-92-MI (25). The authors reported that most serial killing events involved killing of adjacent targets, and they proposed that detachment from one target cell is directly regulated by subsequent attachment to the following target cell.

Our study directly demonstrates for the first time, to our knowledge, that CL protection is due to an inability of perforin to form transmembrane pores on the presynaptic CL membrane during delivery of the lethal hit, and, as a consequence, granzyme-dependent apoptosis of the effector will not occur. Understanding the molecular basis for this protection represents the next challenge. In the past, many hypotheses have been proposed to explain CL protection. Cathepsin B, for example, was proposed to be translocated to the effector cell surface during degranulation and proteolytically cleave any perforin molecules that diffused back onto the effector membrane (26). However, subsequent work
demonstrated that CL survival was not compromised in cathepsin-B null mice, suggesting no direct involvement of this enzyme in CL protection (27). Another possibility is that localized changes in the composition or structure (e.g., membrane topology) of the presynaptic membrane may prevent binding or oligomerization of perforin.

Our novel approach of assessing functional effector-target interactions in real-time and particularly for precisely pinpointing delivery of the lethal hit should also facilitate studies focused on the more proximal events of CL-mediated target cell death: from synapse formation to recruitment, docking, and fusion of cytolytic granules at the IS. Furthermore, the use of extracellular PI as a reporter for target cell permeabilization is a simple and versatile tool that is amenable to any effector-target combination. Using this technique, we have uncovered two critical features of the lethal hit, as follows: a defined focal delivery and rapid target cell membrane resealing. Our study encompassed both primary human and mouse NK and CD8\(^+\) T cells and revealed remarkably consistent kinetics governing delivery of the lethal hit. Our use of peptide-pulsed P815\(^{-/-}\) CTLs as targets has demonstrated that CLs are not intrinsically resistant to perforin permeabilization, yet they are largely protected in the process of secreting these molecules. These findings offer new insight into how CLs protect themselves largely protected in the process of secreting these molecules.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Supplemental Data

Figure Legends

Figure S1. Multiple hits to a single target cell by primary mouse NK cells

Mouse NK cells deliver multiple hits to single target cells; Monitoring the PI fluorescence change over time in a single MC57 target cell, following conjugation of a primary mouse NK cell. Images were acquired every 10 sec. Data represent the fold change in PI fluorescence (F/F0) over time at a site proximal to the immune synapse; Arrows demarcate delivery of 2 distinct lethal hits (Hits 1 and 2).

Movie S1. A primary human NK cell pre-labelled with Fluo-4 killing a HeLa target cell in the presence of 100 µM PI; Fluo4(green)/PI(red)/Brightfield overlay. A time stamp is marked in the bottom right corner.

Movie S2. A primary human CD8+ T cell clone pre-labelled with Fluo-4 killing a B cell target cell in the presence of 100 µM PI; Fluo4(green)/PI(red)/Brightfield overlay. A time stamp is marked in the bottom right corner.

Movie S3. A primary mouse NK cell pre-labelled with Fluo-4 killing a MC57 target cell in the presence of 100 µM PI; Fluo4(green)/PI(red)/Brightfield overlay. A time stamp is marked in the bottom right corner.

Movie S4. A primary mouse CD8+ T cell pre-labelled with Fluo-4 killing a MC57 target cell in the presence of 100 µM PI; Fluo4(green)/PI(red)/Brightfield overlay. A time stamp is marked in the bottom right corner.

Movie S5. A primary human NK cell inflicting multiple hits (3 consecutive) to a single HeLa target cell in the presence of 100 µM PI; PI(red)/Brightfield overlay. A time stamp is marked in the top right corner. The blue box indicates the region within the target cell cytosol (a focal site close to the immunological synapse) that was measured and corresponds to the target cell PI profile shown in Fig. 3A. The text shown in the top left corner, Hit 1, Hit 2 and Hit 3, indicates the timing of each of the hits.

Movie S6. A primary human NK cell inflicting multiple hits (3 consecutive) to a single HeLa target cell in the presence of 100 µM PI; PI(red)/Brightfield overlay. A time stamp is marked in the top
right corner. The blue box indicates a focal site close to the immunological synapse. The text shown in the top left corner, Hit 1, Hit 2 and Hit 3, indicates the timing of each of the hits.

**Movie S7.** A primary human NK cell pre-labelled with Fluo-4 sequentially killing two individual HeLa target cells in the presence of 100 µM PI; Fluo4(green)/PI(red)/Brightfield overlay. A time stamp is marked in the top right corner. Images correspond to target cell PI profiles shown in Fig. 3B.

**Movie S8.** A primary human NK cell sequentially killing three individual HeLa target cells in the presence of 100 µM PI; PI(red)/Brightfield overlay. A time stamp is marked in the top right corner.

**Movie S9.** A primary mouse CD8^+ T cell pre-labelled with Fluo-4 killing a primary mouse Prf1^{-/-} CD8^+ T target cell in the presence of 100 µM PI; Fluo4(green)/PI(red)/Brightfield overlay. A time stamp is marked in the bottom right corner.
Supplementary Figure 1

PI Fluorescence (F/F0)

-30 -10 10 30 50 70 90 110 130 150 170 190 210 230

0 1 2 3 4 5 6 7 8 9 10

Time (sec)

Hit 1

Hit 2