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Imaging of Lytic Granule Exocytosis in CD8⁺ Cytotoxic T Lymphocytes Reveals a Modified Form of Full Fusion

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Abstract

Here we imaged the exocytosis of lytic granules from human CD8⁺ cytotoxic T lymphocytes using rapid Total Internal Reflection microscopy, Lamp-1 tagged with mGFP to follow the fate of the lytic granule membrane, and granzyme A, granzyme B or serglycin tagged with mRFP to follow the fate of lytic granule cargo. Lytic granules were released by full fusion with the plasma membrane, such that the entire granule content for all three cargos visualized was released on a subsecond time scale. The behavior of GFP-Lamp-1 was, however, more complex. While it entered the plasma membrane in all cases, the extent to which it then diffused away from the site of exocytosis varied from nearly complete to highly restricted. Finally, the diffusion properties upon release of the three cargos examined put an upper limit on the size of the macromolecular complex of granzyme and serglycin that is presented to the target cell.

1. Introduction

CTLs kill target cells (e.g. virally-infected cells, tumor cells) by the polarized secretion of lytic granules, a form of secretory lysosome. This process involves a fascinating series of events within the T cell that include complex signaling, rapid reorganization of the cytoskeleton, membrane trafficking, and regulated secretion (for reviews, see [1; 2; 3]). Briefly, CTLs identify target cells through their T cell receptor (TCR), which recognizes in highly specific fashion a cognate peptide presented on the surface of the target cell via a MHC class 1 receptor. TCR engagement leads to a complex array of signaling reactions and protein: protein interactions that drive the process of polarized secretion. This process involves a dramatic rearrangement of membrane proteins in the portion of the T cell's plasma membrane that is in contact with the target cell, resulting in the formation of the immunological synapse (IS). The mature IS is characterized by a central accumulation of TCRs at the "cSMAC" and a peripheral accumulation of the T cell integrin LFA-1 at the "pSMAC", which forms a sealing gasket around the cSMAC via interaction with ICAM in the target cell plasma membrane. During IS maturation, the T cell's interphase microtubule

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array undergoes a dramatic and rapid reorientation such that the centrosome, to which all of the T cells microtubules are attached via their minus ends, is pulled very close to the T cell's plasma membrane at the IS [4]. This centrosome repositioning event is then followed by the microtubule-dependent, minus end-directed movement of lytic granules, which results in their accumulation adjacent to the IS. In terms of the positioning of lytic granules for secretion, these two events, which can actually occur in either order [4; 5], are sufficient for secretion. The final steps of polarized lytic granule secretion involve the docking of the granules to the plasma membrane (which may occur within a specific subdomain of the cSMAC) [6], followed by their calcium- and SNARE-dependent fusion with the plasma membrane, leading to release of the granule's contents (e.g. granzymes, perforin, serglycin) into the cleft between the two cells [7]. By analogy with the SNARE-dependent fusion of synaptic vesicles in neurons, a "priming" step in between the docking and fusion steps may also occur in T cells. The relatively recent identification of molecules involved in the docking, priming and SNARE-dependent secretion of lytic granules has been aided enormously by the characterization of various mouse mutants and of humans with a family of related immunological diseases collectively known as Familial Hemophagocytic Lymphohistiocytosis (FHL) [8]. Amongst other things, these studies have demonstrated that Rab27a on the surface of the lytic granule is required for the docking step and that Munc13-4, a member of a family of proteins known to regulate SNARE-dependent vesicle fusion in neurons, is a critical downstream effector of Rab27a in T cells (see [7] for a recent and comprehensive review of the mechanism of lytic granule secretion).

As interesting as is the mechanism of lytic granule secretion, the mechanism by which the mediators of target cell apoptosis- principally perforin and the two major granzymes (A and B)-gain access to the target cell's cytoplasm is also very interesting, and much more unclear. Various mechanisms have been proposed (for reviews, see [9; 10; 11; 12; 13; 14]). The mechanism first proposed, in which granzymes enter the target cell's cytoplasm through perforin-generated pores in the cell's plasma membrane [15], is no longer favored. Rather, the current model favors a mechanism in which granzymes are taken up as a macromolecular complex with serglycin via receptor-mediated endocytosis (or endocytosis stimulated by a membrane repair pathway downstream from perforin-dependent plasma membrane damage) [16; 17], and then released into the target cell cytoplasm via perforin-dependent escape from the endosome [18; 19; 20; 21; 22]. Indeed, this process of endosomolysis has been compared to the mechanism of entry of certain viruses into the cytoplasm via endocytic uptake [20]. Understanding the physical form of granzyme that is presented to the target cell is also critical to understanding the processes of uptake and target cell death. Granzymes, which have highly basic pI's, are sequestered within lytic granules in a tight ionic complex with serglycin, a highly-processed, negatively-charged proteoglycan possessing extensive chondroitin sulfate-type GAG side chains. Moreover, Froelich and colleagues [19; 20; 21; 22] have presented strong evidence that exocytosed granzymes are presented to target cells as a macromolecular complex in which 30 to 50 granzyme molecules are stably bound to one serglycin molecule of ~300 kDa, yielding complexes in the size range of 1-2 mDa. It is even possible that the contents of the lytic granule remain stably associated on a very large scale following their release (e.g. a large number of serglycin/granzyme complexes stuck together), such as seen in the exocytosis of granules from chromaffin cells [23; 24] and pituitary lactotrophs [25].

The imaging of vesicle secretion using Total Internal Reflection (TIRF) microscopy (or evanescent field microscopy) was pioneered by Almers and colleagues [26; 27]. The power of this form of microscopy is that it provides an enormous increase in signal to noise ratio over even confocal microscopy for events such as exocytosis that occur very close to the surface of the cover slip. The application of TIRF-based imaging to secretion exhibited by a variety of different cell types (that don't include T cells or NK cells, however) has revealed

that exocytosis can occur in several different forms. One common form is full fusion, in which all of the vesicles contents are released into the extracellular space and its resident membrane proteins fully diffuse into the plasma membrane. Various forms of partial fusion have also been identified in which the vesicles' membrane proteins, and in some cases its contents as well, do not fully diffuse prior to termination of the exocytic event. The most dramatic examples of such partial fusions are probably cavicapture in PC12 cells [24] and "kiss-and-run" exocytosis observed in presynaptic nerve terminals [28].

Here we developed methods to visualize lytic granule secretion in human CD8⁺ T cells by TIRF microscopy. Our images, which show this process for the first time in CTLs, shed light on the mechanism of the lytic granule exocytosis in T cells and on the physical nature of the material presented to target cells for uptake.

2. Materials and methods

2.1 Antibodies and reagents

The following mouse monoclonal antibodies were used: clone UCHT1 to human CD3, clone CD28.2 to human CD28, clone CB9 to human granzyme A, and clone 2CF/F5 to human granzyme B (BD Pharmingen, San Jose, CA), clone H4A3 to Lamp-1 (Developmental Studies Hybridoma Bank, Iowa City, IA), clones 7.1 and 13.1 to GFP (Roche Applied Science, Indianapolis, IN), and clone DM1A to α -tubulin (Sigma-Aldrich, St. Louis, MO). The following polyclonal antibodies were also used: anti-DsRed (Clontech, Mountain View, CA) and anti-cathepsin D (Dako, Carpinteria, CA). Alexa Fluor 488- or 568-conjugated goat anti-rabbit IgG, Alexa Fluor 488- or 568-conjugated goat anti-mouse IgG, and Alexa Fluor 647-conjugated goat anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA). HRP-conjugated anti-mouse or anti-rabbit IgG were acquired from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from (Sigma-Aldrich, St. Louis, MO). Protease inhibitor cocktail tablets were obtained from Roche Applied Science. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell preparation and nucleofection

Peripheral blood mononuclear cells (PBMC's) were isolated from lymphocyte apheresis preparations of normal healthy donors from the NIH blood bank by density gradient centrifugation using Ficoll-plaque Plus (GE Healthcare Biosciences, Piscataway, NJ). PBMC's were cultured for 7 days in IMDM supplemented with 10% FCS, antibiotics, and nonessential amino acids, and in the presence of 5 μ g/ml of the lectin phytohemagglutinin (PHA-P; isolated from *Phaseolus vulgaris*) (Sigma-Aldrich, St Louis, MO) and either 20 U/ml of rIL-2 or 100 ng/ml of IL-15. CD8⁺ T cells were purified by negative selection using CD8⁺ T Cell isolation kit (Miltenyi Biotec, Auburn, CA). After purification, cells were maintained for at least for 3 days prior to transfection/imaging in culture medium containing either 20 U/ml of rIL-2 or 100 ng/ml of IL-15. Subpopulation purity, tested by flow cytometry using CD4-PE and CD8-FITC (BD Pharmingen, San Jose, CA), was >95%. Stimulated CD8⁺ T cells were transiently transfected using the Amaxa nucleofection technologyTM (Amaxa, Cologne, Germany). Briefly, 10×10^6 cells were resuspended in 100 μ l of solution from the Nucleofector human T cell kit and mixed with 5 μ g of each cDNA. The cell suspension was then transferred to the provided cuvette and nucleofected using the U-14 pulsing parameter. Immediately after nucleoporation, cells were transferred into wells containing 37°C pre-warmed culture medium in 6-well plates. Cells were cultured for 16-18 h in the presence of rIL-2 or IL-15 and then live cells were isolated by Ficoll-Plaque centrifugation and used for either immunofluorescence or TIRF imaging experiments. The human T cell line TALL-104 (a generous gift of Dr. Luis Montaner, The Wistar Institute,

Philadelphia, PA) was grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine, 15% fetal bovine serum, antibiotics, gentamycin, and 200 IU/ml of rIL-2 at 37°C and 10% CO₂. These cells were transiently transfected using program T-20 and solution V as described above for CD8⁺ T cells.

2.3. Recombinant DNA constructs

The constructs for mRFP-tagged versions of granzyme A (mouse), granzyme B (mouse), and serglycin (mouse) were generated by PCR amplification of full-length cDNAs for mouse granzyme A (GenBank™/EMBL/DDBJ accession number NM_010370 [GenBank]), mouse granzyme B (GenBank™/EMBL/DDBJ accession number NM_013542 [GenBank]), and mouse serglycin (GenBank™/EMBL/DDBJ accession number NM_011157 [GenBank]), followed by in-frame cloning into the *HindIII-EcoRI* sites of vector mRFP1-N1 (Clontech). Mouse granzyme A was also cloned into vector EGFP-N1. To produce mGFP-tagged Lamp1 (lgp120), plasmid EGFP-Igp120 (generously provided by Dr George Patterson, NIH), which contains the full length cDNA sequence for rat Lamp-1 fused at its C-terminus to a repetitive sequence of serine, threonine, and glycine residues as an additional 10-amino acid linker, was digested with *XhoI* and *BamHI* to release the fragment containing Igp120 and the C-terminal linker. This fragment was then purified and ligated into similarly-digested mEGFP-N1. All constructs were confirmed by DNA sequencing.

2.4. Confocal microscopy

Transfected CD8⁺ T cells were plated on coverslips pre-coated with poly-L-lysine (0.01% in deionized water) (Sigma-Aldrich, St Louis, MO). After adherence, the coverslips were washed with PBS and the cells fixed with 4% paraformaldehyde, followed by two quenching steps of 5 min each with 50 mM of NH₄Cl at room temperature. Fixed cells were incubated with the indicated primary antibodies in permeabilization buffer (0.1% saponin, 10% FCS in PBS) for 1h at room temperature, followed by incubation with secondary antibodies conjugated to Alexa fluorophores (Molecular Probes-Invitrogen, Carlsbad, CA). After staining, the coverslips were mounted and images were acquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc, Germany) equipped with a 100X Plan-Apo objective (1.4 NA).

2.5. Total Internal Reflection Fluorescence microscopy

Transfected CD8⁺ T cells were resuspended in complete imaging medium (DMEM without phenol red, supplemented with 5% fetal bovine serum and 20 mM Hepes-HCl pH 7.4) and plated in glass-bottom dishes (35 × 10mm dish with a 12 mm diameter and 0.17 mm thick glass) (Bellco Glass, Inc., Vineland, NJ) that had been pre-coated with a solution containing poly-L-lysine (0.01%), anti-CD3 antibody (10 µg/ml), and anti-CD28 antibody (5 µg/ml). For PMA/ionomycin experiments, cells were suspended in Ca²⁺-free imaging medium (DMEM without CaCl₂ and phenol red, supplemented with 5% dialyzed fetal bovine serum and 20 mM Hepes-HCl pH 7.4). After the cells had attached to the glass and the microscope was set up appropriately for TIRF imaging, calcium, ionomycin and PMA were added to the media to final concentrations of 1 µM, 20 ng/ml, and 1 mM, respectively. TIRF imaging was then initiated at a rate of 30 Hz (30 frames per second, 33 ms/frame), using an exposure time of 21 ms. TIRF imaging was performed on an Olympus IX70 inverted microscope equipped for TIRF. A 60X PlanApo objective (1.45 NA) and a 2.5X relay lens (PE2.5; Olympus) were used. During observation, cells were kept at 37 °C and supplied with humidified air containing 5% CO₂ using a custom-designed stage enclosure. Digital images were captured on an Andor iXon Electron Multiplying CCD (EMCCD) camera (Andor Technology, South Windsor, CT) using MetaMorph software (Molecular Devices Inc., Sunnyvale, CA).

2.6. Planar lipid bilayers

Liposomes were prepared and planar bilayer was formed on coverslip substrates essentially as previously described by Dustin et.al. [29]. Anti-CD3 antibody (OKT3, Biovest International, Tampa, FL) was monobiotinylated and labeled with fluorescent dyes following the protocol of Carrasco et al. A flow chamber was assembled by attaching two layers of double-sided tape to the sides of a glass slide. A mixture of DOPC, biotin-CAP-PE (1% molar ratio), and DOGS NTA (1% molar ratio) liposomes (Avanti Polar Lipids, Alabaster, AL) were deposited in the center of the flow chamber and a glass coverslip, previously washed in piranha solution (mixture of sulfuric acid and hydrogen peroxide), was placed on top of the flow chamber to allow a single planar bilayer to form on the coverslip surface. HEPES buffer saline was then added to the chamber to wash away the remaining liposomes, followed by blocking with 5% (w/v) casein solution. Next, monobiotinylated anti-CD3 antibodies labeled with Alexa-647, as well as streptavidin, were added to the flow chamber to conjugate with biotin-CAP-PE lipids, while His-tagged ICAM-1 was added to conjugate with DOGS NTA lipids. The uniformity and lateral mobility of the lipids in the bilayers was observed by imaging the mobility of the Alexa-647-labeled anti-CD3e antibody.

2.7. Image analyses

Intensity profiles for Lamp1-mGFP and granzyme A-mRFP following degranulation (Figure 6) were determined from a time-sequence stack of images using the software Interactive Data Language (IDL; Research Systems, Boulder, CO). Specifically, granules undergoing exocytosis were visually identified, and from the stack of images, a data set was generated containing x and y coordinates and granule intensities (background subtracted) for each frame. Positions were calculated from the peaks of the parabolas determined by the intensities of the brightest pixel and each of the four immediately adjacent pixels. Total granule intensity was determined in unfiltered images from the total pixel intensity within a circular region (860 nm in diameter, 10 pixel area) around the granule center after the local background was subtracted. The data set was then analyzed to plot the intensity profiles.

2.8. Electrophoresis and immunoblotting

Transfected CD8⁺ T cells were harvested, washed with ice-cold PBS, resuspended in lysis buffer (25 mM Hepes-KOH (pH 7.4), 250 mM NaCl, and 1% (v/v) Triton X-100, supplemented with protease inhibitor cocktail), and lysed by passing the samples 10 times through a 25 gauge needle. Cell lysates were centrifuged at $16,000 \times g$ for 15 min at 4°C, and the soluble fractions were collected. Samples were analyzed by SDS-PAGE (4-20% gradient gels) under reducing conditions and transferred to nitrocellulose. Membranes were immunoblotted using either apolycloal anti-DsRed or a monoclonal anti-GFP antibody. Immunoblots were developed with the corresponding secondary antibodies coupled to horseradish peroxidase using an enhanced chemiluminescence detection kit (ECL, GE Healthcare Bio-Sciences Co., Piscataway, NJ).

2.9. CD8⁺ T cell : target cell conjugate formation

To form T cell : target cell conjugates, 3×10^6 Fas-negative L1210 target cells were washed in Hepes-saline buffer (0.1 M Hepes, 0.15 M NaCl, pH 7.6), and biotinylated with 0.2 mM NHS-LC-biotin (EMD Biosciences, San Diego, CA) in Hepes-saline buffer for 30 min at 4°C. The cells were then washed and coated with streptavidin by incubation with 20 $\mu\text{g/ml}$ of streptavidin (Sigma-Aldrich, St. Louis, MO) in Hepes-saline buffer containing 1% (w/v) BSA for 30 min at room temperature. The cells then were washed with Hepes-saline containing 1% BSA and incubated in the same buffer plus 20 $\mu\text{g/ml}$ of biotinylated anti-CD3 antibody. The cells were then washed twice with complete medium. 2×10^6 CD8⁺ T

cells and an equal number of target (L1210-CD3⁺) cells (each in 50 μ l) were then pre-heated in a water bath for 2 min at 37° with gentle mixing, combined in one tube, spun down for 5 seconds at 300xg, and incubated for 5 min at room temperature. The cell pellet was then gently dispersed, and the cell conjugates transferred to 12-mm poly-L-lysine-coated glass cover slips. The cells were allowed to adhere for a further 3 min and then fixed for 15 min with 4% formaldehyde at room temperature.

3. Results

3.1 Authentic lytic granules in CD8⁺ T cells can be dynamically labeled using granzymes A or B tagged with mRFP but not GFP

Our initial effort to label lytic granules involved the transfection of human CD8⁺ T cells (referred to hereafter simply as CTLs or T cells) with mouse granzyme A tagged with GFP at its C-terminus. Surprisingly, these cells exhibited essentially no GFP fluorescence (data not shown). That said, when we fixed and stained them with an antibody to GFP, we saw clear vesicular staining (Figure 1, Panel A; a cell transfected with free GFP and stained for GFP is shown as a control in Panel B). Moreover, Western blots of whole cell extracts probed with an anti-GFP antibody showed the presence in GFP-granzyme A-transfected cells of a band corresponding in size to GFP-tagged granzyme A (Figure 1, Panel C, lane 1). Together, these results suggested that GFP-tagged granzyme A is synthesized and properly packaged in lytic granules by CTLs, but is not fluorescing for some reason. The most likely explanation for this lack of fluorescence is the low pH inside lytic granules (~5.0) [1], since the fluorescence of GFP exhibits progressive, dramatic quenching as the pH drops below 7.0 [30]. We therefore examined CTLs transfected with granzyme A tagged with mRFP, as red fluorescent proteins like mRFP do not exhibit such pH-dependent quenching. Figure 1, Panels D1 and D2, show that live CTLs expressing mRFP-tagged granzyme A exhibit numerous bright red vesicles of the size consistent with lytic granules (~0.2-0.5 μ m). Moreover, Western blots of whole cell extracts prepared from these cells and probed with an antibody to mRFP showed a band corresponding in size to mRFP-tagged granzyme A (Figure 1, Panel C, lane 2). Identical results were obtained with CTLs transfected with mouse granzyme B tagged at its C-terminus with mRFP (Figure 1, Panels E1 and E2, and Panel 1C, lane 3). These results suggest that lytic granules can be labeled and imaged in live T cells when the enzymes are tagged with mRFP.

To provide more convincing evidence that the red vesicles present in CTLs expressing mRFP-tagged granzymes A and B are indeed authentic lytic granules, we stained transfected cells with antibodies specific for these two enzymes to determine the degree of overlap between the signals for the endogenous, lytic granule-associated granzymes and the transfected, mRFP-tagged versions (note that for these experiments we used anti-granzyme A and B antibodies that recognize the endogenous, human granzyme isoforms but not the transfected, mRFP-tagged mouse isoforms). Figure 2 shows that mRFP-tagged granzyme A co localizes extensively with both endogenous granzyme A (Panels A1-A3) and granzyme B (Panels B1-B3) (see Legend for details). Similarly, Figure 2 shows that mRFP-tagged granzyme B co localizes extensively with both endogenous granzyme A (Panels C1-C3) and granzyme B (Panels D1-D3). Similar results were also seen in TALL-104 cells (a human T cell line) that had been transfected with mRFP-tagged granzyme A and then stained for endogenous granzyme A (Supplemental Figure 1, A1-A4). Correct targeting was also observed for human granzymes, as mRFP-tagged human granzyme B co localizes extensively with endogenous human granzyme A in CTLs (Supplemental Figure 1, B1-B4). Finally, in CTLs expressing mRFP-tagged granzyme B that had been allowed to bind to coverslips coated with anti-CD3/CD28 antibodies, and then stained for microtubules, the red vesicles were seen to accumulate at the minus end of microtubules focused at the centrosome/MTOC (Supplemental Figure 2, Panels A1-A3). Similarly, when CTLs

expressing mRFP-tagged granzyme A were allowed to bind to target cells coated with anti-CD3 antibody, the red vesicles were seen to accumulate at the T cell: target cell interface (Supplemental Figure 2, Panels B1-B3). Together, these results argue strongly that the signals for mRFP-tagged versions of granzymes A and B in living CTLs are reporting authentic lytic granules.

3.2. Lamp-1 tagged with mGFP faithfully reports the limiting membrane of lytic granules in living CTLs

Given our ultimate goal of following the fate of the limiting membrane of the lytic granule during exocytosis, as well as its internal cargos, we sought to label this membrane in living CTLs using human Lamp-1/CD107a labeled at its C-terminus with mGFP (note that this places the GFP moiety on the cytoplasmic face of the lytic granule). This type 1 membrane glycoprotein is a known component of the limiting membrane of lytic granules. Figure 1, Panels F1 and F2, and Figure 3, Panel A1, show that CTLs expressing Lamp-1-mGFP exhibit numerous bright green vesicles of the size consistent with lytic granules. Moreover, these green vesicles stain uniformly with an antibody against cathepsin D, a bona fide lytic granule marker (Figure 3, Panels A1-A3). As expected, Western blots of whole cell extracts probed with an anti-GFP antibody showed the presence in Lamp-1-mGFP-transfected cells of a band corresponding in size to mGFP-tagged Lamp-1 (Figure 1, Panel C, lane 4). Very importantly, in CTLs co transfected with mGFP-tagged Lamp-1 and mRFP-tagged granzyme B, the vast majority of fluorescent vesicular structures present in these cells possessed both tags (Figure 3, Panels B1-B3). Exactly the same result was obtained in CTLs co expressing mGFP-tagged Lamp-1 and mRFP-tagged granzyme A (Figure 3, Panels C1-C3). This is demonstrated most dramatically in the movie from which the still images in Panels C1-C3 were taken (see Supplementary movie 1), where the cells can be seen to contain moving vesicles that uniformly possess a red interior and a green limiting membrane (see also the arrowheads in Figure 3, Panels C1- C3). Finally, in CTLs expressing mGFP-tagged Lamp-1 that had been allowed to bind to coverslips coated with anti-CD3/CD28 antibodies, and then stained for microtubules, the green vesicles were seen to accumulate at the minus end of microtubules focused at the centrosome/MTOC (Supplemental Figure 2, Panels C1-C3). Together, these results argue strongly that mGFP-tagged Lamp-1 and mRFP-tagged granzymes A and B can be used to follow the limiting membrane and contents, respectively, of authentic lytic granules in real CD8⁺ T cells during exocytosis.

3.3. Authentic lytic granules can also be dynamically labeled with mRFP-tagged serglycin

In an effort to create one additional labeled cargo, we tagged the lytic granule matrix proteoglycan serglycin by linking mRFP to the C-terminus of its core, 17-kDa polypeptide. Figure 4, Panel A1, shows that CTLs expressing mRFP-tagged serglycin exhibit numerous bright red vesicles of the size consistent with lytic granules. Moreover, these red vesicles stain uniformly with an antibody against granzyme A (Figure 4, Panels A1-A4). Similarly, in CTLs transfected with mRFP-tagged serglycin and stained with an antibody against Lamp-1, the vast majority of fluorescent vesicular structures present in these cells are double-labeled (Figure 4, Panels B1-B4; note that the green signal for Lamp-1 encircles the red signal for serglycin). Importantly, mRFP-tagged serglycin appears to be processed more or less normally in transfected CTLs. Specifically, Western blots performed using an antibody to RFP on immunoprecipitates of cells expressing mRFP-tagged serglycin that were precipitated using anti-RFP antibody reveal an extensive ladder of cellular products ranging in size up to ~300 kDa (Figure 1, Panel C, lane 6; compare to the control in lane 5). These blots argue that the addition of mRFP to the core serglycin polypeptide does not interfere appreciably with the extensive addition to this core protein of the chondroitin sulfate-type GAG side chains found on the native protein.

3.4. Rapid TIRF imaging of the exocytosis of lytic granules labeled with mRFP-tagged granzyme A and mGFP-tagged Lamp-1 reveals rapid and complete diffusion of the granzyme into the extracellular space and variable degrees of restriction in the diffusion of Lamp-1 into the plasma membrane surrounding the site of exocytosis

We initially imaged CD8⁺ T cells that had been co transfected with mGFP-tagged Lamp-1 and mRFP-tagged granzyme B. The cells were allowed to adhere to glass coated with antibodies to CD3 and CD28 and imaging in TIRF mode was then commenced at a rate of 30 frames per second (33 ms/frame) (see Methods for details). Because these cells do not exhibit robust secretion upon TCR engagement (see below for details), we initially added the calcium ionophore ionomycin and the phorbol ester PMA (final concentrations of 1 μ M and 20 ng/ml, respectively) to stimulate exocytosis. Figure 5, Panels A1 and A2, show a typical still image of lytic granules in the TIRF zone, where many double-labeled lytic granules can be seen. Over the course of the next several minutes, several of these lytic granules underwent exocytosis. An example of one such exocytic event, which involved the lytic granule boxed in Panels A1 and A2, is shown in the still images in Figure 5, Panels B1 and B2 and in two corresponding movies (Supplementary movie 2, which shows the exocytic event at low magnification, and Supplementary movie 3, which shows this event at high magnification). In terms of the signal for granzyme B, it is seen upon exocytosis to diffuse very rapidly into the extracellular space. Indeed, the rate of decay of its signal due to its diffusion into the media was such that it was no longer visible to the eye after five or six frames (i.e. after ~200 ms). This can be appreciated in the sequential still images of this exocytic event shown in Figure 5, Panel B2. This can also be appreciated in the example of the exocytic event presented in Figure 6, Panels A1, A2 and B, and the corresponding movie (Supplementary movie 4), where we measured the actual decay in fluorescence intensity for mRFP-tagged granzyme A following exocytosis. Specifically, the fluorescence profile for granzyme A following exocytosis (Panel 6, Panel A1 and Panel B, red lines), which occurred in between the first and second images shown in Figure 6, Panel A1, shows a rapid increase in width and decline in peak intensity after fusion. As will become clear below, the rapid diffusion of granzyme A upon exocytosis shown in these examples is absolutely typical of all the cargos we imaged (i.e. granzyme A, granzyme B, and serglycin).

In terms of the signal for mGFP-tagged Lamp-1 in the exocytic event shown in Figure 5, the two movies and the still images in Panels B1 and B2 that correspond to this event show that, like granzyme B, it also began diffusing immediately upon exocytosis. That said, its diffusion, which is occurring into the plasma membrane surrounding the site of fusion, was significantly slower than that of the diffusion of granzyme B into the extracellular space. Specifically, the still images in Panel B1 show that while the intensity of the Lamp-1 signal in the plasma membrane continues to decay over time, it remains elevated near the site of exocytosis for more than a second. This can also be appreciated in the example of the exocytic event presented in Figure 6, Panels A1, A2 and B, and the corresponding movie (Supplementary movie 4), where we measured the actual decay in fluorescence intensity for mGFP-tagged Lamp-1 following exocytosis. Specifically, the fluorescence profile for Lamp-1 following exocytosis (Figure 6, Panel A2 and Panel B, green lines), which occurred in between the first and second images shown in Figure 6, Panel A2, shows a much slower increase in width and decline in peak intensity after fusion than seen for granzyme A (Panel B, red lines). This slower rate of diffusion for Lamp-1 into the plasma membrane relative to the rate of diffusion of a non-membrane bound cargo like granzyme A into the media, is to be expected given published values for the rates of diffusion of plasma membrane proteins versus soluble proteins [31]. Importantly, as will become clearer below, these two examples for Lamp-1, which represent the most extensive diffusion of Lamp-1 into the plasma membrane that we observed, still exhibit some degree of retention over many tenths of a second of the Lamp-1-mGFP signal in the immediate vicinity of the site of exocytosis.

Sometimes vesicle-associated signals brighten just before exocytosis [26; 27]. This is due to the movement of the vesicle even closer to the plasma membrane just before fusion, which places it into the strongest portion of the TIRF illumination zone (the strength of this zone decreases exponentially as one moves away from the surface of the glass, such that it is negligible beyond ~ 150 nm (notably less than half the diameter of a typical lytic granule). While we did not attempt to quantitate the number of exocytic events that did and did not exhibit in a clear fashion such transient brightening just before membrane fusion, we did see examples of both situations (data not shown). In those instances where the lytic granule did not brighten just before fusion, we assume it was already morphologically docked (ergo very close to the plasma membrane). In terms of other aspects of vesicle dynamics prior to fusion, in other systems what is typically seen is a continuum between the vesicle fusing soon after its appearance in the TIRF zone (presumably having just been delivered to the plasma membrane by a microtubule- or actin-dependent motor protein) and the vesicle having been present for a significant period of time in the TIRF zone (possibly having already docked and waiting for the priming and fusion steps) (see for example [32; 33]). While we did not quantitate in a precise way the distribution of lytic granule exocytic events across this continuum, we did see examples that fall all along it. Some of these will become apparent in the specific examples presented below.

3.5. The majority of lytic granule exocytic events exhibit a dramatic retention of Lamp-1 in the region of the plasma membrane immediately surrounding the site of exocytosis

In about 80% (39 out of 48) of the lytic granule secretion events we observed using ionomycin and phorbol ester on glass, the diffusion of mGFP-tagged LAMP-1 into the plasma membrane surrounding the site of exocytosis was significantly retarded. A typical example of one such exocytic event is shown in the still images in Figure 5, Panels C1 and C2, and in the corresponding movie (Supplementary movie 5). In this event, the signal for Lamp-1-mGFP (Panel C1) remains very intense for more than four seconds in an area of the plasma membrane that is centered on the site of exocytosis and has a diameter of ~2 μ m. In most of these cases (although not always; see Supplementary movie 6), we also observed a dark “hole” of variable size in the middle of the retained Lamp-1-mGFP fluorescence that was centered at the site of membrane fusion, giving rise to what looks like a ring of Lamp-1-mGFP fluorescence around the fusion site (Panel C1). Indeed, such rings of fluorescence are also apparent in the examples of Lamp-1 diffusion shown in Figure 5, Panel B1, and Figure 6 Panel A2 (note, in particular, the intensity profile in Figure 6, Panel B (green lines) for the spreading of the Lamp-1 signal for the exocytic event shown in Figure 6, Panels A1/A2, which shows a dip or minimum in the middle), although the rings in both of those events are fainter and shorter-lived than the one in Figure 5, Panel C1. Examples of very slow diffusion of a vesicle membrane protein into the plasma membrane following exocytosis, as well as the concomitant appearance of hole or minimum in fluorescence centered at the site of fusion, have also been reported by others. For example, Allersma et al. [32] found that in ~10% of exocytic events exhibited by PC12 cells, the vesicle membrane protein VAMP exhibits an intense ring of fluorescence with a hole in the center that persists in the plasma membrane at the site of fusion for many seconds. While we did not attempt to determine the basis for the exclusion of Lamp-1 fluorescence that gives rise to the “hole” in lytic granule exocytic events, in other contexts such “holes” have been attributed to incomplete flattening of the vesicle membrane into the plasma membrane (such that it remains above the TIRF zone and is therefore not visible [32]). Moreover, neither we nor others [32] know what causes the variable retardation in the diffusion of vesicle membrane proteins into the plasma membrane in a subset of exocytic events. One possible role for such restricted diffusion could be to facilitate the subsequent endocytic reuptake of the vesicle membrane protein (ref). Importantly, we never saw exocytic events that are

consistent with cavicapture [24], an exocytic mechanism that directly facilitates the reuptake of both membrane cargo and vesicle contents (see Discussion).

3.6. Rapid TIRF imaging of lytic granules labeled with mRFP-tagged serglycin A reveals that this matrix proteoglycan also undergoes very rapid diffusion into the extracellular space upon exocytosis

To image the exocytosis of serglycin, T cells were co transfected with mGFP-tagged Lamp-1 and mRFP-tagged serglycin. An example of an exocytic event involving the release of serglycin, which actually involved the sequential fusion of two adjacent lytic granules, is shown in the still images in Figure 6, Panels C1 and C2 (the first lytic granule is marked with red arrowheads, while the second one is marked with green arrowheads; also see the brackets to the left of Panel C1), and the corresponding movie (Supplementary movie 7). In terms of the signal for serglycin, it is seen in both exocytic events to diffuse very rapidly into the extracellular space much like granzyme A. Indeed, the rate of decay of its signal due to its diffusion into the media was such that it was no longer visible to the eye after five or six frames (i.e. less than ~200 ms). Overall, therefore, the behavior of mRFP-tagged serglycin upon degranulation is essentially identical to that of the granzymes, i.e. both exhibit complete diffusion into the extracellular environment in less than a second.

These two sequential secretion events also reveal two interesting features of lytic granule exocytosis that have been observed previously by TIRF-based imaging of dense core vesicle secretion in PC12/chromaffin cells [32; 33]. The first is that vesicles sometimes undergo sub-micron sized, lateral movements within several hundred milliseconds of undergoing exocytosis. The second of the two lytic granule events in Figure 6, Panels C1 and C2, involves one such lateral movement. Specifically, this lytic granule moves laterally about ~0.5 μm ~0.6 seconds before undergoing fusion (see the brackets to the right of Panel C1, and the green arrowheads that point to this second granule during its movement). The second characteristic is that vesicles often fuse at a site on the plasma membrane that was used previously by another vesicle (a “preferred” site) [33]. This behavior is also exhibited by this second lytic granule, as its lateral movement placed it exactly at the site where the first lytic granule had fused moments before. This movement can be best appreciated in Figure 6, Panel D, where the first, sixth and twelfth still images (top to bottom) from Panel C1 are placed in register. This type of behavior has been interpreted as evidence of the recruitment of the vesicle to a site where the SNARE machinery has already assembled [33].

3.7. The basic characteristics of lytic granule secretion are not altered when ionomycin and phorbol ester are omitted, when T cells are induced with PHA/IL-15 instead of PHA/IL-2, and when the stimulatory anti-CD3 antibody is free to diffuse in a planar lipid bilayer instead of being bound to glass

All of the examples of lytic granule secretion shown so far were obtained in the presence of ionomycin and phorbol ester to increase the frequency of fusion events. That said, we did observe a few exocytic events without adding these compounds (i.e. with only TCR engagement to stimulate secretion), although these events occurred with ~15-fold lower frequency. An example of one such exocytic event is shown in the still images in Figure 7, Panels A1 (mGFP-tagged Lamp-1) and A2 (mRFP-tagged granzyme B) and in the corresponding movie (Supplementary movie 8). Importantly, all of the basic properties of lytic granule secretion described above (e.g. rapid, complete diffusion of cargo, variable degrees of diffusion of Lamp-1 into the plasma membrane surrounding the site of fusion) were observed in these events, arguing that the addition of ionomycin and phorbol ester does not alter the intrinsic mechanism of lytic granule secretion.

In an effort to obtain CTLs that might exhibit more robust TCR-dependent secretion, CD8⁺ T cells were produced by incubation of PBMCs with PHA/IL-15 instead of PHA/IL-2. These T cells were readily transfectable and did exhibit fairly robust dumping of Lamp-1 into their plasma membrane (as measured by FACS analysis) upon incubation with anti-CD3 coated beads (data not shown). In a limited number of experiments, however, we saw no obvious increase in the frequency of TCR-dependent exocytic events on anti-CD3/CD28-coated glass surfaces (and in the absence of ionomycin/phorbol ester) as compared to PHA/IL-2-induced CTLs.

In a final effort to obtain more robust secretion downstream from TCR ligation, we engaged IL-15-induced CTLs using anti-CD3 antibody that was free to diffuse in a synthetic planar bilayer as opposed to being bound to glass. Such presentation might indeed increase the extent of TCR signaling, and thus TCR-dependent secretion, because TCRs on bilayer-engaged cells are able to readily assemble into signaling microclusters, unlike the situation on glass. While we did not see in a limited number of experiments any obvious increase in the frequency of exocytic events in bilayer-engaged, IL-15-induced T cells (done in the absence of ionomycin and phorbol ester), we did find that the characteristics of the few secretion events we did see were very similar to what we observed on glass. An example of a fusion event on the bilayer is presented in Figure 7, Panels B1-D2. Specifically, Panels B1 and B2 show a still image of the lytic granules in the TIRF zone of a bilayer-engaged T cell, while Panels D1 and D2 show sequential still images of the exocytic event for the lytic granule boxed in Panels B1/B2 (see also Supplementary movie 9). Analyses of the intensities of the signal for Lamp-mGFP for this lytic granule (see Figure 7, Panel C and the brackets above Panel D1) revealed several of the common characteristics shown or described above, including entry into the TIRF field shortly before undergoing fusion, a significant brightening just before exocytosis (presumably during the docking/priming steps), very rapid diffusion of the cargo (mRFP-tagged granzyme B) upon fusion (Panel D2), and a much slower diffusion of Lamp-1-mGFP into the plasma membrane surrounding the site of fusion (Panels C and D1).

4. Discussion

This work represents, to our knowledge, the first ever visualization of lytic granule exocytosis by CD8⁺ cytotoxic T cells using bonafide cargos and a membrane marker. Indeed, an earlier study argued that lytic granules cannot be labeled dynamically in living T cells using GFP-tagged granzyme A because it does not target properly (ref). Here we show that GFP-tagged granzymes most likely target properly, but that their fluorescence is probably quenched by the low pH inside the granule. By switching to mRFP, whose fluorescence is not quenched significantly by low pH, we were successful at labeling lytic granules in living CD8⁺ T cells. A similar conclusion as regards the efficacy of labeling of granzymes with red fluorescent proteins instead of GFP was reached recently by Bird and colleagues [34], who examined the expression of tagged versions of granzyme B in Cos cells and YT cells, an NK cell line. Importantly, the extensive overlap that we saw between the expressed, tagged versions of the three cargos we studied- granzyme A, granzyme B, and serglycin- with their endogenous counterparts and cohorts argues strongly that we are labeling authentic lytic granules.

The overall conclusion from this study as regards the mechanism of exocytosis of lytic granules from cytotoxic T cells is that the degranulation event involves a modified form of full fusion. We call it modified full fusion because while the three cargos we visualized undergo in every case complete release into the media on a subsecond time scale, the diffusion of LAMP-1 into the plasma membrane surrounding the site of exocytosis was in most cases significantly retarded. Why this is the case is unclear, but such slowing/

restriction in the diffusion of a vesicle membrane protein upon exocytosis has been observed in other systems. For example, the diffusion of the vesicle membrane protein GFP-VAMP-1 away from the site of secretion in PC12 cells is similarly restricted in ~10% of fusion events [32]. Interestingly, the retention of exocytosed Lamp-1 in the plasma membrane at the center of the immunological synapse of natural killer cells undergoing degranulation has also been reported [35]. One possible role for such restriction could be to facilitate the endocytic reuptake of the Lamp-1 [35]. Whatever its role, we note that our demonstration of this modified form of full fusion in no way contradicts the common practice of gauging lytic granule secretion by measuring the appearance of Lamp-1/CD-107 on the surface of T cells, although the degree to which Lamp-1 is then taken back up into the cell by endocytosis should be taken into consideration [36].

Comparing our results to those in other systems provides some insight into the physical state of lytic granule contents following exocytosis. Most notably, evidence has been presented in several cells types including pituitary lactotrophs [25] that the contents of their dense core secretory granules can remain largely aggregated following release into the media. Presumably, the expansion and dissolution of the granule matrix must be so slow in these cases that granule contents remain particulate for seconds to minutes after exocytosis. The characterization of cavicapture-type exocytosis, which is quite common in chromaffin cells, has provided insights into another form of such behavior [24; 37]. In cavicapture, the chromaffin granule forms a fusion pore with the plasma membrane that can persist for minutes. Importantly, granules exhibiting cavicapture never fully collapse into the plasma membrane. Rather, at some point in time (up to many minutes later) the fusion pore closes, recreating an internal granule. Importantly, while certain cargos leave connected, open granules on a subsecond time scale following establishment of the fusion pore (most notably catecholamine and most of the peptide NPY), other cargo (most notably tissue plasminogen activator (tPa)) leave the connected granule very slowly. Indeed, it is common for only a small fraction of tPa to leave the connected granule over time periods up to tens of seconds. In fact, images of granules that contain florescent tPa and are undergoing cavicapture (i.e. are open to the media) have the appearance in TIRF of intact, intracellular granules. Multiple lines of evidence argue strongly that the very slow egress of tPa from such granules is due to its very slow desorption from the granule matrix. Indeed, when imaging is performed in elevated extracellular calcium, which slows matrix expansion and dissolution, even peptide NPY can show significant retention in connected, open granules [24; 37]. In striking contrast to these studies, all three of the lytic granule cargos we visualized (one of which was the matrix proteoglycan serglycin) underwent in every case complete and very rapid (i.e. subsecond) diffusion into the extracellular fluid. These observations argue strongly against the possibility that the contents of lytic granules are secreted in a particulate form, or that lytic granules exhibit some form of cavicapture. Rather, our imaging of mRFP-tagged serglycin argues that the lytic granule matrix undergoes very rapid dissolution to smaller, rapidly-diffusing macromolecular complexes (or individual molecules; see below) upon release from the cell.

One potential caveat to the above conclusion is that by tagging the core serglycin protein with mRFP, we have interfered significantly with its posttranslational modification (specifically the extensive addition of GAG-side chains), such that the physical properties of the matrix in transfected cells, including the degree to which serglycin molecules self associate/aggregate, is abnormal. While we cannot rigorously exclude the possibility that this has occurred to some extent, it seems unlikely that the defect is very significant given our immunoprecipitation data showing that mRFP-tagged serglycin is highly processed with GAG side chains. We conclude, therefore, that the rapid diffusion of mRFP-tagged serglycin into the media that we see upon degranulation probably reflects the behavior of endogenous, native serglycin. We also note that the very rapid diffusion of mRFP-tagged serglycin is

precisely mirrored by the rapid diffusion of the two granzymes following exocytosis. These observations provide further evidence that our results reflect the true behavior of the matrix and its bound cargos upon degranulation. Again, one caveat to this conclusion is that the behaviors of mRFP-tagged granzymes upon exocytosis might not reflect that of the native proteins because tagging the molecules with mRFP might alter their interaction with serglycin. While we cannot entirely exclude this possibility, we think it is unlikely given that the P_i of mRFP is close to neutral. As such, it would not be expected to interfere appreciably with the strong electrostatic interaction between the highly-basic granzymes and the highly-acidic, chondroitin sulfate-type GAG side chains of processed serglycin.

While our results can be used with reasonable confidence to exclude that possibility that the contents of lytic granules are released in a very large, stable, particulate form (as seen, for example, in lactotrophs), they cannot be used to address more specific and interesting questions as to the exact size of the macromolecular complexes that exist at the moment of secretion and thereafter. As described in the Introduction, Froelich and colleagues [19; 21; 22] have presented clear evidence that each exocytosed serglycin molecule may have upwards of 30-50 stably-bound granzyme molecules attached to it (giving a complex in the size range of 1.5 mDa). How slowly granzymes might then desorb from such a complex is unknown. Resolving these and other issues based on measuring diffusion rates in TIRF imaging is not possible because we cannot image fast enough to obtain numbers that might distinguish the diffusion of individual granzyme molecules from that of granzymes/serglycin macromolecular complexes in the size range of 1-2 mDa.

One problem that we were not able to overcome in this study was that the CD8⁺ human T cells used throughout most of this study, which were differentiated from PBMCs using the lectin PHA and IL-2, did not appear to exhibit robust TCR-dependent secretion. This is despite the fact that these cells did exhibit a reasonable degree of exocytosis in suspension upon incubation with anti-CD3 coated beads, as gauged by the presentation of Lamp-1/CD107 on their surface (measured by FACS sorting; data not shown). Indeed, most of our imaging of exocytosis in these lectin-induced cells was performed in the presence of ionomycin and phorbol ester to “push” secretion. That said, we did image some degranulation events in the absence of these inducers, and their characteristics were indistinguishable qualitatively from that of cells in the presence of ionomycin and phorbol ester. We also asked if the degree of TCR-dependent secretion could be improved if we presented the anti-CD3 antibody such that it was bound to and freely diffusing in a planar lipid bilayer, as opposed to being randomly anchored to glass. Such presentation might indeed increase the extent of TCR signaling (and thus TCR-dependent secretion) because, unlike the situation on glass, TCRs on bilayer-engaged cells are able to assemble readily into signaling microclusters. While we did not see any obvious increase in the frequency of exocytic events in bilayer-engaged T cells in a limited number of experiments, we did find that the characteristics of the secretion events we did see were very similar to what we observed on glass.

We also attempted to create CD8⁺ T cells through other means of differentiation that might exhibit more robust TCR-dependent secretion. While differentiation using anti-CD3 antibody yields potent CD8⁺ T cells (as judged by very robust presentation of Lamp-1/CD-107 on their surface following incubation with anti-CD3-coated beads; data not shown), these cells were totally refractive to Amaxa nucleofection (100% die upon nucleofection). The other approach we tried, where CD8⁺ cells were produced by incubation of PBMCs with PHA and IL-15, was more successful in that the cells were transfectable and did exhibit fairly robust dumping of Lamp-1 into the plasma membrane upon incubation with anti-CD3 coated beads (data not shown). In a limited number of experiments, however, we saw no obvious increase in the frequency of TCR-dependent exocytic events on either glass surfaces

or bilayers as compared to IL-2-induced CTLs. Future efforts will need to seek other possible solutions to the problems we encountered here as regards the TCR-dependency of degranulation in transfected CD8⁺ T cells.

In summary, we have shown here that lytic granules in CD8⁺ cytotoxic T cells can be dynamically labeled and their exocytosis imaged by fast TIRF microscopy. By following both lytic granule cargos and a resident membrane protein, we can conclude that lytic granules are released by a modified form of full fusion, and that the contents of the granules, including its matrix proteoglycan, undergo very rapid diffusion immediately upon release. Clearly, lytic granule contents do not persist in very large, particular form after release, as has been seen for dense core granule released from pituitary lactotrophs [25]. Similarly, lytic granules contents do not persist in highly-aggregated form upon fusion pore opening, as is seen quite commonly for tPa and the chromaffin granule matrix protein chromagranin during cavi capture in PC12 cells [24; 37]. Rather, we think that the physical nature of lytic granule contents immediately after degranulation is probably some form of macromolecular complex on the order of what Froelich and colleagues [19; 21; 22] have suggested, i.e. individual or small groups of serglycin molecules with many granzyme molecules attached that can diffuse very rapidly. These complexes represent the physical form of granzyme (and possibly perforin as well) that is taken up by the target cell via receptor mediated endocytosis.

Our results also complement a very recent study by Long and colleagues [38] that used rapid TIRF microscopy to image lytic granule secretion by natural killer (NK) cells. Similar to CTLs, lytic granule secretion in NK cells can occur by essentially complete fusion. Unlike CTLs, however, a significant fraction of fusion events in NK cells appear to be of the “kiss and run” type, where lytic granule membrane components do not diffuse into the plasma membrane at all upon establishment of the fusion pore, and the essentially-intact lytic granule subsequently detaches from the plasma membrane and moves back into the cell. Despite this and other important differences between these two studies, both indicate that rapid TIRF imaging of lytic granule secretion in immune cells should be very useful for answering many important questions regarding the biology of this granule. For example, this approach can be used to further explore the current, rather complex model of lytic granule maturation, in which three different membrane compartments must fuse together to create mature, fusion-competent lytic granules at the last moments before degranulation [39]. This approach should also be useful for defining in more detail the functional roles of a growing number of molecules (e.g. Rab27a, Munc13-4, Slp1, Slp2, etc) that have been linked to the docking, priming and SNARE-dependent fusion of lytic granules [7].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- We imaged lytic granule exocytosis in cytotoxic T cells by rapid TIRF microscopy
- Cytotoxic T cells degranulate using a modified form of full fusion
- Three different lytic granule cargos exhibit very rapid diffusion following release
- A lytic granule membrane protein usually exhibits restricted diffusion upon release
- Lytic granule contents are not released in particulate form

Fig. 1. mRFP-tagged granzyme A, but not GFP-tagged granzyme A, exhibits a vesicular pattern in T cells. Panels A and B show representative T cells that had been transfected with GFP-tagged granzyme A (A) or GFP only (B) and then subjected to immunofluorescence staining with anti-GFP antibody. Panel C shows Western blots of whole cell extracts of T cells that had been transfected with GFP-tagged granzyme A (lane 1; anti-GFP antibody), mRFP-tagged granzyme A (lane 2; anti-RFP antibody), mRFP-tagged granzyme B (lane 3; anti-RFP antibody), mGFP-tagged Lamp-1 (lane 4; anti-GFP antibody), mRFP vector only (lane 5; anti-RFP antibody), and mRFP-tagged serglycin (lane 6; anti-RFP antibody). The molecular weight markers (see hash marks) are (top to bottom) 180, 115, 82, 64, 49, 37, 26, 19, 16, and 6 kDa (lanes 1, 5 and 6) and 190, 109, 79, 60, 47, and 35 kDa (lanes 2-4). Panels D1, E1, and F1 show representative T cells that had been transfected with mRFP-tagged granzyme A (D1), mRFP-tagged granzyme B (E1), or mGFP-tagged Lamp-1 (F1). Panels D2, E2, and F2 show the corresponding phase image. Mag bars: 2.1 μm (A), 3.6 μm (B), and 2.6 μm (D1, E1), and 3.3 μm (F1).

Fig. 2. mRFP-tagged granzyme A and granzyme B target to authentic lytic granules. Panels A1 and A2, and Panels B1 and B2, show representative T cells that had been transfected with mRFP-tagged granzyme A (A1 and B1) and then fixed and stained for either endogenous granzyme A (A2) or endogenous granzyme B (B2). The corresponding overlaid images are shown in Panels A3 and B3. Visual inspection of these and other samples revealed that $94 \pm 3\%$ ($n=8$ cells) of all labeled granules contained detectable signals for both granzyme A-mRFP and endogenous granzyme A, and $91 \pm 4\%$ ($n=8$ cells) for both granzyme A-mRFP and endogenous granzyme B. Panels C1 and C2, and Panels D1 and D2, show representative T cells that had been transfected with mRFP-tagged granzyme B (C1 and D1) and then fixed and stained for endogenous granzyme A (C2) or endogenous granzyme B (D2). The corresponding overlaid images are shown in Panels C3 and D3. Visual inspection of these and other samples revealed that $92 \pm 4\%$ ($n=8$ cells) of all labeled granules contained detectable signals for both granzyme B-mRFP and endogenous granzyme A, and $95 \pm 3\%$ ($n=8$ cells) for both granzyme B-mRFP and endogenous granzyme B. Importantly, the anti-human granzyme A and B antibodies used here recognize the endogenous, human granzyme

isoforms present in these human CTLs but not the transfected, mRFP-tagged mouse isoforms. Also note that the intensity of the yellow signal in the overlaid images underestimates the actual extent of overlap in the two signals because of differences in the intensities of the two signals. Mag bars: 3.0 μm (A3, B3, C3, and D3).

Fig. 3. mGFP-tagged Lamp-1 targets to authentic lytic granules. Panels A1 and A2 show a representative T cell that had been transfected with mGFP-tagged Lamp-1 (A1) and the fixed and stained for cathepsin D (A2), a bonafide lytic granule marker. The overlaid image is shown in A3. Visual inspection of these and other samples reveals that $91 \pm 3\%$ ($n=8$ cells) of all labeled granules contained detectable signals for both markers. Panels B1-B3 show a representative T cell that had been co-transfected with mGFP-tagged Lamp-1 (B1) and mRFP-tagged granzyme B (B2). The overlaid image is shown in B3. Visual inspection of these and other samples reveals that $94 \pm 3\%$ ($n=8$ cells) of all labeled granules contained detectable signals for both markers. Panels C1-C3 show a video still from supplementary movie 1 of a T cell co-expressing mGFP-tagged Lamp-1 (C1) and mRFP-tagged granzyme A (C2). The corresponding overlaid image is shown in C3. Visual inspection of these and other samples reveals that $94 \pm 3\%$ ($n=8$ cells) of all labeled granules contained detectable signals for both markers. Note that in this high mag image, the lytic granules appear as red balls with a green limiting membrane (see arrowheads). Also note that the intensity of the yellow signal in the overlaid images underestimates the actual extent of overlap in the two signals because of differences in the intensities of the two signals. Mag bars: $4.1 \mu\text{m}$ (A3), $6.2 \mu\text{m}$ (B3), and $2.0 \mu\text{m}$ (C3).

Fig. 4.

mRFP-tagged serglycin targets to authentic lytic granules. Panels A1 and A2, and Panels B1 and B2, show representative T cells that had been transfected with mRFP-tagged serglycin (A1 and B1) and then fixed and stained either for granzyme A (A2) or Lamp-1 (B2). The corresponding overlaid (Panels A3 and B3) and phase (Panels A4 and B4) images are also shown. Visual inspection of these and other samples reveals that $91 \pm 4\%$ ($n=8$ cells) of all labeled granules contained detectable signals for both mRFP-tagged serglycin and granzyme A, while $94 \pm 4\%$ ($n=8$ cells) of all labeled granules contained detectable signals for mRFP-tagged serglycin and Lamp-1. Note that the intensity of the yellow signal in the overlaid images underestimates the actual extent of overlap in the two signals because of differences in the intensities of the two signals. Mag bar: $2.6 \mu\text{m}$ (A4 and B4).

Fig. 5.

Imaging of lytic granule exocytosis by fast TIRF shows that mRFP-tagged granzymes are released completely and diffuse rapidly into the extracellular space, while mGFP-tagged Lamp-1 exhibits variable degrees of restriction in its diffusion into the plasma membrane. Panels A1 and A2 show a cluster of lytic granules in the TIRF field that are double-labeled with Lamp-1-mGFP (A1) and granzyme B-mRFP (A2). Panels B1 and B2 show still images of the fates of these two markers for the lytic granule boxed in A1/A2. See also supplementary movies 2 and 3. Panels C1 and C2 show the fates of Lamp-1-mGFP and granzyme B-mRFP for a lytic granule secretion event where the diffusion of Lamp-1 into the plasma membranes surrounding the site of exocytosis is very restricted. The times for each frame shown in Panels B1/B2 and C1/C2 (in seconds) are shown underneath. Mag bar: 2.9 μm (A1).

Fig. 6.

Quantitation of the spread of fluorescence of granzyme A-mRFP and Lamp-1-mGFP in a typical lytic granule fusion event, and the fate of mRFP-tagged serglycin following exocytosis. Panels A1 and A2 show still images of the fate of granzyme A-mRFP and Lamp-1-mGFP in a typical lytic granule fusion event, while Panel B shows the change in fluorescence intensity (based on a line scan across the center of the site of exocytosis for the event shown in A1/A2) over time (listed in seconds to the right) for granzyme A-mRFP (red lines) and Lamp-1-mGFP (green lines). See also supplementary movie 4. Panels C1 and C2 show still images of the fate of serglycin-mRFP (SG-mRFP; C1) and Lamp-1-mGFP (C2) for two lytic granules that fuse in close succession (see the bracket to the left labeled Event 1

for the lytic granule marked with a red arrowhead, and the bracket labeled Event 2 for the lytic granule marked with a green arrowhead). The times for each frame shown in Panels C1/C2 (in seconds) are shown to the right. Note that, just before undergoing fusion, the second granule moves to the exact position where the first granule fused (see the bracket to the left labeled Granule Migration). This movement can be better appreciated in Panel D, where the first, sixth and twelfth still images (top to bottom) from Panel C1 are placed in register, and the position where the first granule fused is marked with a yellow vertical line, while the position where the second granule resided just before moving and fusing is marked with a white vertical line. See also supplementary movie 7.

Fig. 7.

The characteristics of granule secretion events are unchanged in the absence of ionomycin and phorbol ester and when the stimulatory anti-CD3 antibody is presented in a planar bilayer. Panels A1 and A2 show still images of the fates of Lamp-1-mGFP (A1) and granzyme B-mRFP (A2) for a typical secretory event witnessed in the absence of ionomycin and phorbol ester. The times for each frame (in seconds) are shown underneath. See also supplementary movie 8. Panels B1 and B2 show a cluster of lytic granules in the TIRF field of an PHA/IL-15-induced CTL that had been co transfected with Lamp-1-mGFP (A1) and granzyme B-mRFP (A2) and engaged using a synthetic planar bilayer containing anti-CD3 antibody that is free to diffuse in the bilayer. Panels D1 and D2 show still images of the fates of these two markers for the lytic granule boxed in B1/B2. Panel C shows the change over time in Lamp-1-mGFP fluorescence intensity for the lytic granule shown Panels D1/D2. The areas of interest in this plot that are discussed in the text are show above. The positions of these interesting events are also noted by brackets above Panels D1/D2. The times (in seconds) for each frame shown in Panels D1/D2 and depicted graphically in Panel C are shown underneath. See also supplementary movie 9. Mag bar: 4.2 μ m (B1).