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NK Cell Lytic Granules Are Highly Motile at the Immunological Synapse and Require F-Actin for Post-Degranulation Persistence

Emily M. Mace,* Winona W. Wu,†,1 Tina Ho,‡,1 Shaina S. Mann,† Hsiang-Ting Hsu,§ and Jordan S. Orange*§,*

The formation of a dynamic, actin-rich immunological synapse (IS) and the polarization of cytolytic granules toward target cells are essential to the cytotoxic function of NK cells. Following polarization, lytic granules navigate through the pervasive actin network at the IS to degranulate and secrete their toxic contents onto target cells. We examined lytic granule motility and persistence at the cell cortex of activated human NK cells, using high-resolution total internal reflection microscopy and highly quantitative analysis techniques. We illustrate that lytic granules are dynamic and observe substantial motility at the plane of the cell cortex prior to, but not after, degranulation. We also show that there is no significant change in granule motility in the presence of Latrunculin A (which induces actin depolymerization), when added after granule polarization, but that there is a significant decrease in lytic granule persistence subsequent to degranulation. Thus, we show that lytic granules are highly dynamic at the cytolytic human NK cell IS prior to degranulation and that the persistence of granules at the cortex following exocytosis requires the integrity of the synaptic actin network. The Journal of Immunology, 2012, 189: 000–000.

Natural killer cells are the cytotoxic effectors of the innate immune system and detect virally infected, tumorigenic, or otherwise stressed cells, using germline-encoded activating receptors. Upon encountering a susceptible target, NK cells can mediate directed cytotoxicity following the formation of an immunological synapse (IS) and exocytosis of specialized secretory lysosomes, which contain the lytic effector molecules perforin and granzyme (reviewed in Ref. 1). The steps leading to NK cell exocytosis are highly regulated, as human NK cells are prearmed with constitutively mature lytic granules and need not undergo further activation or expansion to kill (2, 3).

NK cell lytic granule exocytosis is preceded by the dynein-dependent convergence of granules to the microtubule organizing center (MTOC) and subsequent polarization of the MTOC and granules to the IS (4). Once polarized, lytic granules undergo docking and fusion with the NK cell membrane, after which their contents can be released upon the target cell. A dynamic actin cytoskeleton is required for multiple aspects of cytotoxicity and IS maturation, including lytic granule polarization and degranulation (5, 6). Furthermore, the association of granules with actin filaments in a pervasive actin network suggests a role for actin specifically in granule trafficking immediately prior to exocytosis (5, 7, 8). The actin motor protein myosin IIA, which is also required for degranulation, is found at both the IS and the surface of lytic granules, and inhibition or loss of myosin IIA function results in impaired delivery and movement of granules at the plasma membrane (9, 10).

To address the question of lytic granule delivery and the role of the cytoskeleton in this process, we sought to determine the behavior of granules at the plasma membrane of activated human NK cells. We used total internal reflection fluorescence microscopy (TIRFm) because it is designed for accurate visualization of objects within 150 nm of a glass surface. Thus, with TIRFm we studied only those granules present at the NK cell plasma membrane in living cells, using vital labeling with LysoTracker Red and a constitutively expressed lysosomal activation marker protein 1 (LAMP1)-fluorescent reporter. We have previously designed the LAMP1-pHlourin reporter to identify degranulation events in living cells because the construct allows for the sorting of LAMP1-pHlourin to lytic granules, with the pHlourin contained within the granule (5). At baseline lytic granule acidic pH, the pHlourin does not fluoresce, but when the granule pH changes to a more neutral pH upon degranulation, the pHlourin can be excited to fluoresce green. Use of these technologies allowed us to identify and track individual granules both before and after exocytosis. We found that individual granules underwent dynamic, undirected movement at the plasma membrane prior to, but not following, fusion and release of granule contents. Surprisingly, depolymerization of the actin cytoskeleton with Latrunculin A (LatA) did not affect pre-exocytosis lytic granule movement. The integrity of the actin cytoskeleton, however, was required for persistence of granules following fusion, defining a specific interplay between NK cell lytic granules and synaptic actin as well as a role for synaptic actin in degranulation.

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Abbreviations used in this article: IS, immunological synapse; LAMP1, lysosomal activation marker protein 1; LatA, Latrunculin A; MFI, mean fluorescent intensity; MTOC, microtubule organizing center; NA, numerical aperture; TIRFm, total internal reflection microscopy.
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MOTILITY OF LYTiC GRANULES AT THE NK CELL SYNAPSE

Materials and Methods

Cell lines
The NK92 pHuorin-LAMP1 cell line (5) and YTS GFP-actin (11) cell lines were generated previously. All NK cell and 721.211 and K562 target cell lines were maintained as described (12).

Live cell confocal microscopy
For imaging of NK cells with target cells, NK cells (effectors) were suspended in RPMI 1640 10% FBS at a concentration of 10⁶ cells per milliliter and incubated with 10 μM LysoTracker Red DND-99 at 37°C for 30 min, then washed and resuspended in media. Effector were mixed at a 2:1 ratio with target cells that had been preincubated for 5 min with 5 μg/ml CellMask Plasma Membrane Stain (Invitrogen). Conjugates were plated in Lab-Tek No.1.0 borosilicate chamber slides (VWR) that had been coated with 5 μg/ml anti-CD48 (BD clone TU145) for 30 min at 37°C, then washed three times to adhere target cells and thus facilitate imaging. Effectors and target cells were incubated for 30 min; then SYTOX Blue was added to a final concentration of 1 μM. For NK92 LAMP1-phuorin experiments, conjugates were imaged on a Leica SP8 laser scanning confocal microscope with 100× 1.45 numerical aperture (NA) objective. Excitation was provided by a UV laser at 405 nm and tunable white light laser at 488, 561, and 647 nm. Emission was detected with HyD detectors, and images were collected in a single z-plane at one frame per minute for 60–150 min. Data were acquired with LAS AF software (Leica), then exported to Velocity (PerkinElmer) for analysis. For NK92 and YTS conjugates, cells were imaged in a single plane on a Zeiss Axioplan Observer Z1 fluorescence microscope with a 63× 1.4 NA objective and Yokogawa CSU-10 spinning disk with excitation by 405-, 488-, 568-, and 647-nm lasers in a LMM5 laser combiner unit (Spectral Applied Research). Images were acquired for 90–130 min at a rate of one frame per minute, using Velocity software (PerkinElmer). Temperature was maintained at 37°C with a ΔT dish heater and lid (Biophotons).

Live cell TIRFm
For NK92 lytic granule motility studies, pHuorin-LAMP1 NK92 cells were suspended in dye-free RPMI 1640 10% FBS media at a concentration of 7.5 × 10⁶–10⁶ cells per milliliter and incubated with 100 nM LysoTracker Red DND-99 (Invitrogen) at 37°C for 30 min, washed, and resuspended in media. ΔT culture dishes (Biophotons) were coated with 5 μg/ml anti-NKp30 and -CD18, incubated at 37°C for 1 h or 4 overnight, washed with PBS, and prewarmed with dye-free RPMI 1640 10% FBS media. NK92 cells were plated on ΔT culture dishes immediately prior to imaging, and temperature was maintained at 37°C throughout the experiment with a ΔT culture dish heater and lid (Biophotons).

Cells were imaged in a single z-axis plane 10–15 min after addition to imaging chambers through an APoTIRFs, oil immersion 60× 1.49 NA objective using an Olympus IX-81 spinning disk confocal microscope with a rectilinear charge-coupled device C9100 camera. Excitation was provided by 480-nm (Spectra-Physics) and 561-nm (Cobalt) diode lasers through an LMM5 charge-coupled device C9100 camera. Excitation was provided by 480-nm a rear-mounted TIRF illuminator and a Hamamatsu electron-multiplying objective using an Olympus IX-81 spinning disk confocal microscope with Scientific) was added to cells in the imaging chamber, but only after 10 min imaging, and temperature was maintained at 37°C throughout the ex-

Results
Polarization of lytic granules to the IS precedes their synaptic persistence prior to target cell death
We sought to analyze the dynamics of NK cell lytic granule convergence, polarization, and target cell death in live NK cells. YTS GFP-actin cells were loaded with LysoTracker Red DND-99, which selectively labels acidified organelles and fluoresces at 568 nm in acidic pH. Susceptible 721.221 target cells were labeled with CellMask to discern them from effector cells, and NK–target cell conjugates were imaged every 60 s for 60–120 min via live cell spinning disk confocal microscopy. Imaging was performed in the presence of SYTOX Blue nucleic acid stain, which does not permeate the membrane of living cells and thus identifies dying cells within the population. Following initial contact with target cells, NK cell granules were observed to rapidly converge to the MTOC, as has been previously reported (4). This was then followed by polarization of granules to the IS (Fig. 1A, top panel, Supplemental Video 1). Although granule polarization occurred rapidly, the initiation of target cell death as marked by SYTOX entry was not visualized until approximately one-half-hour later (Fig. 1A, bottom panel, Supplemental Video 1). Analysis of 10 conjugates supported this observation, with the mean time of granule polarization measured as 41.5 ± 12 min (Fig. 1B) and SYTOX Blue entry being first observed at a mean time of 61.5 ± 14 min (Fig. 1C). During this time, granules remained converged at the MTOC and continued to be visible, suggesting they were not undergoing degranulation or recycling. Thus, granule polarization preceded initiation of target cell death by ~20 min.

To ensure that these observations were not specific to the YTS cell line, these same parameters were studied in a second and distinct NK cell line. We imaged NK92 GFP-tubulin NK cells conjugated to susceptible K562 targets, using the same strategy as above. Similar to YTS-mediated target cell apoptosis, NK92-mediated target cell death was delayed following granule polarization. Quantitation of six cell conjugates confirmed this observation, with a mean time of granule polarization of 24.2 min (Fig. 1D) and a mean time of initiation of target cell death of 54.2 min (Fig. 1E). Thus, in a distinct NK cell line, the elapsed time be-
tween granule polarization and onset of target cell death was also delayed and, with these NK cells, was ~30 min.

Finally, to visualize the polarization of granules relative to target cell death with reference to NK cell degranulation on a single-cell level, we used NK92 cells expressing a pH-sensitive GFP mutant fluorescent protein (pHlourin) fused to LAMP1 to visualize individual degranulation events. LAMP1 (CD107a) is sorted to lytic granules and is often used as a marker of degranulation when found on the cell surface (13, 14). At acidic pH within lytic granules, pHlourin-LAMP1 is not excited by green wavelengths, but following degranulation and exposure to neutral pH, the pHlourin exhibits fluorescence characteristics of GFP and green emission is observed (5, 15). To identify granules prior to degranulation, NK92 LAMP1-pHlourin cells were loaded with LysoTracker Red. Therefore, individual granules could be tracked over time and degranulation events observed by a transition from red fluorescence, derived from LysoTracker Red in acidified organelles, to green fluorescence derived from the neutralization of pH and subsequent activation of LAMP1-pHlourin.

With this strategy for visualizing both granules and degranulation events, NK92 LAMP1-pHlourin NK cells were conjugated with K562 targets and imaged live using spinning disk confocal microscopy. As seen in Fig. 2 and Supplemental Video 2, granule polarization was marked by movement of LysoTracker Red-loaded granules to the cell membrane. This occurred relatively rapidly in the example shown, with maximum polarization by 16 min following 10 min of contact (Fig. 2B). The progress of granules toward the IS was followed by NK cell degranulation, as marked by a transition from green to red granule fluorescence at the plasma membrane. Initial, transient degranulation events were seen as early as 6 min following granule polarization (Fig. 2B). These early degranulation events, however, did not persist, with each lasting <5 min. The early transient degranulations were followed by later events beginning at 25 min that were sustained until the end of imaging.
Target cell death, as evidenced by a surrogate measure, membrane blebbing, began at \( \sim 40 \) min (2). Analysis of four time-lapse conjugates showed relatively consistent times of polarization, as seen in Fig. 1, but highly variable times of initial degranulation events, demonstrated by the appearance of LAMP1-pHlourin at the membrane (Fig. 2C).

Lytic granules are dynamic prior to, but not following, degranulation at the NK IS

To better understand the nature of the polarized lytic granule, its transition to degranulation, and behavior thereafter, we recapitulated the NK cell IS using activating Abs immobilized on a glass surface, thus allowing for orientation of the NK cell synapse in the \( XY \) plane to enable high-resolution imaging. We used Ab to the natural cytotoxicity receptor NKp30 and CD18, the \( \beta_2 \) subunit of LFA-1, to activate NK cells for degranulation. Together, these signals induce polarized secretion of lytic granule contents and degranulation in the \( XY \) plane (5, 16). This approach also allows for visualization of degranulation events by TIRFm, which provides high-resolution imaging limited to the membrane-proximal 100 nm of cells bound to Ab-coated glass. As above, we used NK92 LAMP1-pHlourin NK cells labeled with LysoTracker Red to analyze individual cells prior to, during, and after degranulation at the NK cell plasma membrane.

Cells were imaged using two-color TIRFm at the interface between the cell and the glass in the \( XY \) plane over time in heated imaging chambers following 10 min of contact. Numerous polarized lytic granules, indicated by red fluorescence derived from LysoTracker Red labeling, were identified at the plane of the IS. In contrast, degranulation events (red to green color transition) were visualized less frequently at the plane of the cell cortex and observed within the TIRF field over time (Fig. 3A, Supplemental Video 3).

To quantify granule behavior after polarization to the IS, individual LysoTracker Red-labeled lytic granules undergoing degranulation were measured and tracked in the \( XY \) plane with TIRFm. Prior to degranulation, an overlay of these tracks at the synapse showed seemingly random, yet highly dynamic, movement of granules navigating the cell cortex (Fig. 3B). Tracks of the same granules following degranulation, however, demonstrated greatly reduced motility, as seen by shorter tracks with less displacement (Fig. 3C). This observation was further quantitated by measurement.
Strikingly, pre-degranulation, granules had longer track lengths, with a mean length of 10.2 ± 4.2 μm, compared with 1.0 ± 0.7 μm following degranulation (Fig. 4A). The mean pre-degranulation velocity for granules was 0.005 μm/sec ± 0.002, whereas any post-degranulation granule velocity was almost 10-fold less, 0.0005 ± 0.0003 μm/sec (Fig. 4B). In addition to track length, granules prior to degranulation had a greater displacement from their point of origin, 1.2 ± 0.6 μm, when compared with post-degranulation, 0.2 ± 0.1 μm (Fig. 4C). Finally, the rate of this displacement in granules prior to degranulation, 0.0005 ± 0.0005 μm/sec, was greater than that following degranulation, 0.0001 ± 0.00007 μm/sec (Fig. 4D). Thus, lytic granules are dynamic and demonstrate considerable nondirected motility at the NK cell IS.
while navigating the cell cortex before degranulation. Following degranulation, this motility is significantly reduced.

**Degranulation events do not predict pre-degranulation motility and persistence**

It has been observed that many more NK92 lytic granules approximate the IS than degranulate (5). As a result, a significant number of polarized granules that navigate the IS are not exocytosed. Because it was unclear if the dynamics of these granules would be the same as the dynamics of those that degranulated, we investigated the dynamics of synaptic lytic granules that do not degranulate. Individual LysoTracker Red-labeled lytic granules that did not result in degranulation events were distinguished by a consistent, unchanging red fluorescence. These were measured and tracked in the $XY$ plane throughout the duration of imaging until they disappeared from the TIRF field (Fig. 5A; Supplemental Video 4). Relative to LysoTracker Red-labeled NK92 granules that degranulate, those that do not degranulate were similarly dynamic. An overlay of lytic granule approximation tracks showed considerable nondirected movement (Fig. 5B). These lytic granules had lateral mobility that was not significantly different from the lateral mobility in those that go on to degranulate. The track velocity of granules that did not degranulate was $0.004 \pm 0.001 \text{ m/sec}$, which was not significantly different from the mean of those that degranulate (Fig. 6A). Similarly, the mean track length of $11.5 \pm 3.3 \mu$m, for granules that did not degranulate, was comparable to the track length of those that degranulated (Fig. 6B). The mean displacement and displacement rate of granules that did not degranulate were $1.3 \pm 0.9 \mu$m and $0.0004 \pm 0.0003 \mu$m/sec, respectively, which were also not significantly different from the displacement and displacement rate for those that degranulated (Fig. 6C, 6D). Thus, irrespective of whether approximation leads to degranulation, lytic granules have characteristic dynamics at the NK-activating IS.

However, considerable variability in the duration of granule persistence within the synapse was observed. To determine whether degranulation influenced the persistence of LysoTracker Red-labeled lytic granule tracks, the previously identified approximation events were compared. The difference between the time of visibility of LysoTracker Red signal of lytic granules that did not degranulate, $45.8 \pm 12.1$ min, and the time of visibility of signal for those that did, $39.0 \pm 14.2$ min, was not significant (Fig. 6E). The length of visibility of signal at the synapse also did not affect granule dynamics. A subset of granules were visible for $>55$ min and until the end of imaging (and thus did not degranulate or withdraw). These prolonged nondegranulated events were identified and analyzed throughout their lifetime. Despite their prolonged presence at the synapse, these granules had kinetics similar to the kinetics of both those that degranulated and those that withdrew earlier (Supplemental Fig. 2).

Degranulation is accompanied by a significant decrease in the velocity of lytic granules prior to the degranulation event (Fig. 4). To determine if all granules undergo similar changes in velocity, we plotted the velocity of granules against time. As shown in Fig. 6F, all granules have a reduction in velocity over time. This finding suggests that the mechanism of arrest of granules at the synapse is independent of that of degranulation. In addition, lytic granules at the cell cortex that do not degranulate have time of persistence and lateral mobility at the cortex that are statistically indistinguishable from these same measures in those that do degranulate. Thus, synaptic granule arrest precedes, but does not mandate, degranulation.

**Cortical actin integrity is required for post-degranulation persistence, but not pre-degranulation motility**

Actin cytoskeleton integrity and remodeling are required for NK cell cytotoxicity. Treatment with the actin polymerization inhibitor

![FIGURE 5. Lytic granules that do not degranulate show normal synaptic motility. Lytic granules demonstrating no observed degranulation were analyzed. (A) A representative NK92 lytic granule cropped from image sequence is shown at 5-min intervals following 10 min of contact. LysoTracker Red (red) track denoting all observed time points is shown in final 55-min image. Scale bar, 1 \mu m. (B) Overlay of LysoTracker Red tracks of 10 lytic granules over four separate experiments. Representative granule track from (A) is depicted in bold (red).](http://www.jimmunol.org/)
LatA rapidly depolymerizes NK cell cortical actin filaments (5) and abrogates cytotoxic function (6, 17). We used LatA to investigate the role of actin in supporting synaptic granule motility at the cortex. Specifically, NK92 LAMP1-phlourin cells were treated with 10 μM LatA or vehicle control (DMSO) 10 min after being added, and the lytic granules were observed via TIRFm. Surprisingly, pre-degranulation motility of individual granules in LatA-treated cells was intact and similar to that in control-treated cells (Fig. 7A, 7B; Supplemental Videos 5, 6). Despite LatA treatment, granules continued to demonstrate motility prior to, but not after, degranulation (as measured by a shift from red to green fluorescence) (Fig. 7A, 7B, final panel). Measurement of 12–18 tracks from LatA-treated and control-treated cells, normalized to the values of the DMSO control, demonstrated no significant differences in lytic granule track length, velocity, and total displacement attributable to LatA (Fig. 7C).

The major difference following LatA treatment, however, was in regard to granule lifetime. Granules for which termination of degranulation was observed (as marked by the disappearance of green fluorescence) were selected and the timespan of the granule measured both pre- and post-degranulation. No difference was found in the measured lifetime of red fluorescence of granules in LatA-treated cells, 1.1 ± 0.6 arbitrary units, compared with normalized control treated cells (1.0 ± 0.9). This observation suggested that the time to degranulation was not affected by LatA. However, when compared with DMSO-treated control cells, degranulated granules in LatA-treated cells persisted for significantly less time, as measured by a shorter timespan of green fluorescence, 0.6 ± 0.4 arbitrary units, compared with control, 1.0 ± 0.6 (normalized) (Fig. 7D, p = 0.04). This finding suggests that actin integrity is required for the maintenance of degranulation events at the plasma membrane.

**Loss of actin integrity leads to increased granule area**

Although the green fluorescence of some granules was visible throughout the complete imaging time course (60–80 min), other granules ceased to be visible in the TIRF field following degranulation. This finding suggested that the “disappearing” degranulation events were withdrawn into the cell and therefore out of the field of TIRF. Alternatively, the lumenal LAMP1-phlourin of degranulated events was diluted by diffusion throughout the membrane adjacent to the site of degranulation and was thus falling below the threshold for detection by imaging. To begin to distinguish between these possibilities, the persistence of granules was plotted using a Kaplan–Meier survival curve (Fig. 8A). As imaging continued for variable times following degranulation, some imaging sequences ended with granules still visible (vertical ticks, Fig. 8A). However, compared with granules from DMSO-treated cells, those from LatA-treated cells demonstrated shortened survival, indicating decreased persistence following degranulation, with a median time of 13.7 min for 50% survival, compared with 28.8 min for control-treated cells. This observation suggests that the shortened survival of those that degranulated was a feature of their being withdrawn, thus implying a role for actin in maintaining the degranulated granule at the cortex prior to its withdrawal into the cell.

The difference in persistence, however, still did not exclude a more rapid loss of green fluorescence following degranulation in LatA-treated cells owing to faster dissipation of granule contents and/or the granule membrane. To help discriminate between the two models (dissipation and withdrawal), the area and MFI of granules in LatA- or DMSO-treated cells was measured at 10-s intervals following degranulation (phlourin fluorescence). Specifically, were there to be increased dissipation in the absence of...
actin integrity, there should be an increased area of pHluorin signal observed immediately after degranulation in the LatA-treated cells. A significantly greater sum fluorescent intensity (MFI*area) of granules from DMSO-treated cells up to 40 min post-degranulation was seen at almost all time points tested (Fig. 8B; \( p < 0.0001 \) by the Mann–Whitney \( U \) test). This occurred despite a slightly greater area of granules from LatA-treated cells, as shown in Fig. 8C. This difference in area was significant by the Mann–Whitney \( U \) test over 40 min (\( p < 0.0001 \)). The difference, however, may have been partially due to fewer granules remaining, thereby weighting the results in favor of the few remaining larger granules. Truncation of the measurements at an earlier time (20 min post-degranulation) resulted in loss of significance in granule area, suggesting the difference may not be physiologically relevant (\( p = 0.4; \) Supplemental Fig. 1A). Of interest, truncation at 20 min did not affect significance of the difference in sum fluorescent intensity (\( p < 0.0001; \) Supplemental Fig. 1B). This finding suggests that over all time points measured, granules from control-

![Image of Figures 7A, 7B, 7C, 7D](http://www.jimmunol.org/Downloadedfrom)
Results from four independent experiments are shown. Fluorescent intensity with 3 SD above background as a cutoff. DMSO (solid black) or LatA (dashed red). Granule boundaries were de-

Our results in activated NK cells, taken together with those pre-

Discussion

In this study, we have carefully determined the nature of lytic granule kinetics and degranulation in NK cells, using pH sensors in combination with TIRFm. We have shown that NK cell lytic granules undergo undirected yet highly dynamic motility prior to, but not immediately preceding or following, degranulation. Importantly, once granules are delivered to the IS, actin integrity is not required for granule motility at the cell cortex immediately pre-

FIGURE 8. The synaptic actin network is required for persistence of degranulation. (A) The lifetime of lytic granules post-degranulation in NK cells treated with LatA or DMSO control. Time points reflect amount of time elapsed post-degranulation (marked by the appearance of LAMP1-phluorin), with vertical drops indicating disappearance of the granule from the TIRF field. Vertical ticks indicate granules persisting to the end of the imaging sequence. (B) Sum fluorescent intensity of lytic granules in NK92 cells expressing pHluorin-LAMP1. Cells were treated with DMSO (solid black) or LatA (dashed red) as per Fig. 7. Note that sum fluorescent intensity is a function of both the area and mean fluorescent intensity of a lytic granule. (C) Area of the observed lytic granules in cells treated with DMSO (solid black) or LatA (dashed red). Granule boundaries were defined using fluorescent intensity with 3 SD above background as a cutoff. Results from four independent experiments are shown.
viously reported in otherwise resting NK cells (20), suggest that NK cells may employ multiple mechanisms of granule movement, including activation-specific, actin-independent movement. Interestingly, in neutrophils, disruption of actin by cytochalasin D treatment does not prevent movement of their granules in the TIRF plane at the membrane but, rather, seems to promote the accumulation of these granules at the exocytic zone. This observation suggests that actin remodeling is not required for lateral movement but instead may be required for clearing the way for granules through cortical actin (23). This idea may reflect commonality between cells of the innate immune system and their mechanism of secretion. With that being said, Latrunculin B treatment does not result in increased numbers of granules at the plasma membrane in resting human NK cells (20), and we did not observe any gross accumulation of granules at the activated NK IS in the presence of LatA (data not shown).

Although not required for granule movement prior to exocytosis, however, actin dynamics affect the post-degranulation process, as we have shown. Specifically, actin is required for the persistence of granules at the membrane following degranulation. This requirement is consistent with a role for F-actin in the expulsion of granule contents, the tethering of granules at the cortex, or the retention of granule contents at the plasma membrane to prevent diffusion. Our data are consistent with the first two of these models (Fig. 9A). Although some difference in the area of granules from LatA-treated cells was observed at later time points, which could support a role for actin in the prevention of lateral diffusion, this may have been due to the bias of the measurement of a few granules that had survived to this time. At all times, however, there was a significant difference in fluorescent intensity of granules following degranulation from LatA-treated cells that did not correspond to an increase in area. This finding suggests that actin is not required to simply act as a fence preventing the diffusion of granule contents, but may serve as a platform for the generation of force, resulting in the squeezing of contents from the granule, as well as act as a tether to retain the granule at the cortex. This model is consistent with that of other cell types in which actin has been considered both a barrier to, and permissive of, granule exocytosis. In PC12 chromaffin cells, treatment with LatA results in a more rapid expulsion of granule contents, which would be consistent with our observation of shorter persistence of fluorescence post-degranulation (24). Together, these results suggest a requirement for force in fully extracting lytic components from granules, which contain a dense core, including extracellular matrix components such as proteoglycans (25, 26). It should also be noted that, although we did not observe a difference in granule velocity or displacement, we did see a significant difference between control and LatA-treated cells in displacement rate, with LatA-treated cells having a significantly lower pre-degranulation displacement rate. This observation also supports a model in which actin may function in the tethering or catching of granules. Finally, actin remodeling may be required for the formation of permissive clearances in the cortex. However, although not quantified here, no observable defect was noted in the number of granules.

**FIGURE 9.** Models for the role of F-actin in granule persistence and the varying behavior of lytic granules at the NK cell cortex. (A) A LysoTracker Red-loaded LAMP1–pHlourin–expressing granule is depicted approaching within the cell cortex, nearing a region within the F-actin network suitable for membrane access [as previously demonstrated (5, 7)] (1). As docking and fusion occur (2), F-actin acts as a tether to help anchor the granule at the membrane, although in both cases fusion results in the activation of LAMP1–pHlourin and the subsequent appearance of green fluorescence. In addition, actin reorganization is likely to act in the generation of force to aid in the focused expulsion of granule contents (as supported by greater area*intensity of pHlourin-LAMP1 in control- compared with LatA-treated cells) (3) and the continued persistence of the degranulating granule at the cortex, which we would propose is a feature of the interaction of the granule with the local F-actin network (4). This is followed by the approximation of the granule to the cell cortex (3), movement (4), and then one of the outcomes depicted: (i) immediate exocytosis, (ii) reduced movement and degranulation, (iii) immediate withdrawal from the cortex, or (iv) reduced movement before withdrawal.

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A Model for the role of F-actin in degranulation

With F-actin polymerization:

1. LAMP1-pHlourin
2. F-actin
3. F-actin
4. LAMP1-pHlourin

Without F-actin polymerization:

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B Model for different observed granule kinetics at the cortex

1. Granule approximation
2. F-actin tethering
3. Membrane approximation
4. Granule movement

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i) Degranulation
ii) reduced movement at the cortex and then degranulation
iii) Withdrawal
iv) reduced movement at the cortex and then withdrawal
that reached the TIRF plane in LatA-treated cells, consistent with prior reports (20).

The observation that granules undergo dynamic motility for such a substantive amount of time is somewhat surprising. The seemingly undirected nature of the movement may reflect a need for the granule to travel to a point of suitable actin clearance to be secreted. It may also indicate a requirement for granules to seek docking domains for membrane tethering and fusion. The observation that granules undergo slowing and arrest of movement regardless of whether they degranulate (Fig. 6F) suggests that a sustained presence at the cell cortex ultimately results in arrest for most granules. As described in the model in Fig. 9B, however, granules that polarize to the synapse seem to have multiple outcomes. These include degranulation, persistence, or withdrawal. The relationship between these models is unclear, particularly as motility does not predict degranulation or lack thereof.

In conclusion, our results demonstrate a previously unreported dynamic motility of NK cell granules prior to, but not following, degranulation. To our knowledge, they include the first demonstration of degranulation in a living NK cell in conjugation with its target cell. They also show a surprising role for actin dynamics in post-degranulation, but not pre-degranulation, granule behavior at the activating NK cell synapse. This finding implies that in addition to serving a role early after synapse formation in enabling NK cell lytic granule localization toward the target cell, the synaptic actin meshwork facilitates the compression of lytic granules. Thus, actin would have a function in the optimal extrusion of lytic granule contents by providing a platform for the generation of force by which the granule contents are fully emptied.

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Disclosures
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References