

Integrin-Dependent Organization and Bidirectional Vesicular Traffic at Cytotoxic Immune Synapses

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Supplemental Experimental Procedures

Cells and Reagents

Transfection and maintenance of *Drosophila* S2 cells have been described (Barber and Long, 2003). 293T cells were obtained from ATCC (American Type Culture Collection, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). A 6-histidine tag was incorporated at the C-terminus of the complete extracellular domains of ICAM-1, CD48, and ULBP1 in order to attach these ligands onto lipid bilayers in the proper orientation. Human IgG1 Fc was tagged with (His)₆ at the N-terminus in order to leave the C-terminal region exposed, as it would be when intact IgG is bound to cells. The extracellular coding sequence of human ICAM-1 was generated by a Pfu-based PCR using a cDNA template (Barber and Long, 2003) and the primers 5'-cccaagcttctcagcctcgetatggctcc-3' and 5'-tccccgcggtcataccggggggagagca-3'. The PCR product digested with HindIII and SacII was ligated into HindIII and SacII-digested vector pcDNA6/V5-His B (Clontech). As a result, the extracellular region of ICAM-1 (ending in sequence-VTVNVLSPRYE) was fused to a 21 amino acid linker, including a V5 epitope (PRFEGKPIPPLLGLDSTRTG) preceding the C-terminal 6 histidines. Histidine-tagged CD48 and ULBP1 were constructed using primers that included 6 histidine codons. Primers for ULBP1-(His)₆ were 5'-ccgctcgagcggaccatggcagcggccgccc-3' and 5'-gctctagagctcaatggtgatggtgatgatgtggggccagagagggtgg-3'. A ULBP1 cDNA was first obtained by RT-PCR of RNA from the cell line K562. Primers for CD48-(His)₆ were 5'-ccgctcgagctggaaggaagcatgtgctccag-3' and 5'-gctctagagctcaatggtgatggtgatgatgccgggcccagggtacag-3'. The cDNA of CD48 has been

IL), conjugated with Alexa Fluor 647, and further purified by Sephacryl S-200 size-exclusion HPLC (Figure S7A). The Fab preparation contained a single population of molecules with an apparent molecular weight of 50 kDa, which corresponds to monomeric Fab. The Fab purity and integrity was further confirmed by Coomassie Blue staining and immuno-blotting.

Lipid Bilayers

Planar bilayers were formed by fusing small liposomes droplets with clean glass coverslips as described (Brian and McConnell, 1984). Planar phospholipid bilayers were formed between a coverslip and the microaqueduct slide in a Bioptechs parallel plate flow chamber-FCS2 (Bioptechs, Butler, PA) as described (Dustin and Springer, 1988). Histidine-tagged proteins, either unconjugated or conjugated with Alexa dyes, were added to the bilayers. The density of ligands on the lipid bilayer was determined by comparison with standard FITC Silica beads (Quantum MESF Fluorescence intensity calibration beads, Bangs Laboratories, Inc. IN). Unless specified otherwise, densities on the bilayers were: 150 molecules/ μm^2 for CD48, 250 molecules/ μm^2 for ICAM-1, 200 molecules/ μm^2 for ULBP1, and 200 molecules/ μm^2 for IgG1 Fc. Degranulating NK cells were detected on lipid bilayers carrying 40 Fc molecules/ μm^2 but not at 8 Fc molecules/ μm^2 (data not shown).

Microscopy

TIRF imaging was performed on an Olympus inverted IX-81 microscope equipped with 100× 1.45 N.A. TIRF objective (Zeiss, Plan-Fluar 100×/1.45 oil) and an electron-multiplier charge-coupled device (Photometrics Casade II:512, Roper Scientific). TIRF illumination was provided by the 488 nm line of an Argon laser (150W, Laser Pysics, UT, USA) and 568/647 nm line of a Krypton-Argon laser (Dynamic Laser, Salt Lake City, UT, USA). The hardware on the microscope was controlled by Metamorph software (Molecular Devices). The penetration depth of evanescent field was calculated as ~87 nm, assuming a cell refractive index of 1.37. Confocal images were acquired on a Zeiss LSM 510 Meta confocal microscope using a plan apochromat 63× 1.4 N.A. oil immersion objective. All images were acquired at 37°C using an FSC2 chamber (Bioptechs, Butler, PA). Images were analyzed with Image Pro Plus 6.1 (Media Cybernetics, Silver Spring, MD). Graphs were produced with Igor Pro (Wavemetrics, Lake Oswego, OR). Single molecule analysis was performed by Matlab (Mathworks) code. Calculation of the penetration depth in the TIRF microscopy setup was made according to the equation:

$$d = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2 \theta - n_2^2}}$$

in which λ is the wavelength of light and n_1 represents the refractive index of the glass coverslip ($n_1 = 1.518$) (Axelrod, 2001). θ is the critical angle. The n_2 is the refractive index of the aqueous buffer solution or internal cellular components ($n_2 = 1.33$ to 1.37 , typically $n_2 = 1.37$). The number of Alexa-labeled Fab molecules bound to LAMP-1 that was exocytosed by NK cells was quantified relative to the intensity of single Fab

molecules, which could be detected during transient binding to the lipid bilayer. The distribution of intensity of individual spots was determined by an automated process. Each fluorescent spot that exceeded a set intensity threshold in the image area surrounding the NK cells (Figure S8A) was fit to a 2-D Gaussian function (Figure S8B-S8E). Only spots with a full width at half-maximum (FWHM) that deviated from the point spread function (PSF) of our setup (FWHM = 2.9 pixel = 435 nm) by less than 40% were included for further analysis. Thereby, only focused signals were selected, analyzed in a histogram and fit to a lognormal distribution (Figure S7B, right panel). The mode of this fit yields the intensity that most of the fluorescently labeled Fab molecules had in the experiment. Intensities of individual LAMP-1 clusters within areas covered by NK cells in the same recording were determined by a similar procedure (Figure S8F), but selected manually rather than by the automated process. In addition, as the local density of clusters can lead to the fusion of two nearby fluorescent objects to a single one, the Gaussian function was allowed to adopt an elliptical shape. Finally, the intensity value of each cluster was divided by the intensity value of single Fab molecules in the same recording, to obtain the number of Fab molecules located at that cluster.

NK-Target Cell Conjugates

Conjugate formation between NK cells and S2 cells was described (Barber and Long, 2003). Briefly, 1×10^6 resting NK cells were conjugated to S2 cell expressing ICAM-1 or co-expressing ICAM-1, CD48, and ULBP-1 at a 1:2 ratio in suspension in RPMI1640 medium containing 5% FBS at 37°C. In ADCC assays, S2 cells were coated with rabbit

anti-S2 serum at a 1×10^{-4} dilution and incubated for an additional 30 min at room temperature. S2 cells were washed three times with PBS and placed over poly-L-lysine-coated slides. After removal of unbound S2 cells, NK cells were added to the slides at 37°C for different times, fixed with 4% paraformaldehyde at room temperature for 20 min, and permeabilized. For perforin staining, cells were stained with anti-perforin mAb (δ G9, Pierce Chemical Co.) and revealed with Alexa Fluor-conjugated goat anti-mouse IgG2b secondary antibody (Invitrogen). For MTOC staining, cells were permeabilized and stained with primary antibody against β -tubulin (clone tub2.1, Cy3-conjugated; Sigma-Aldrich). To visualize internalized LAMP-1, 0.016 μ M Alexa Fluor 555-labeled CD107a Fab was included during incubation of NK cells with S2 insect cells.

Supplemental Figures:

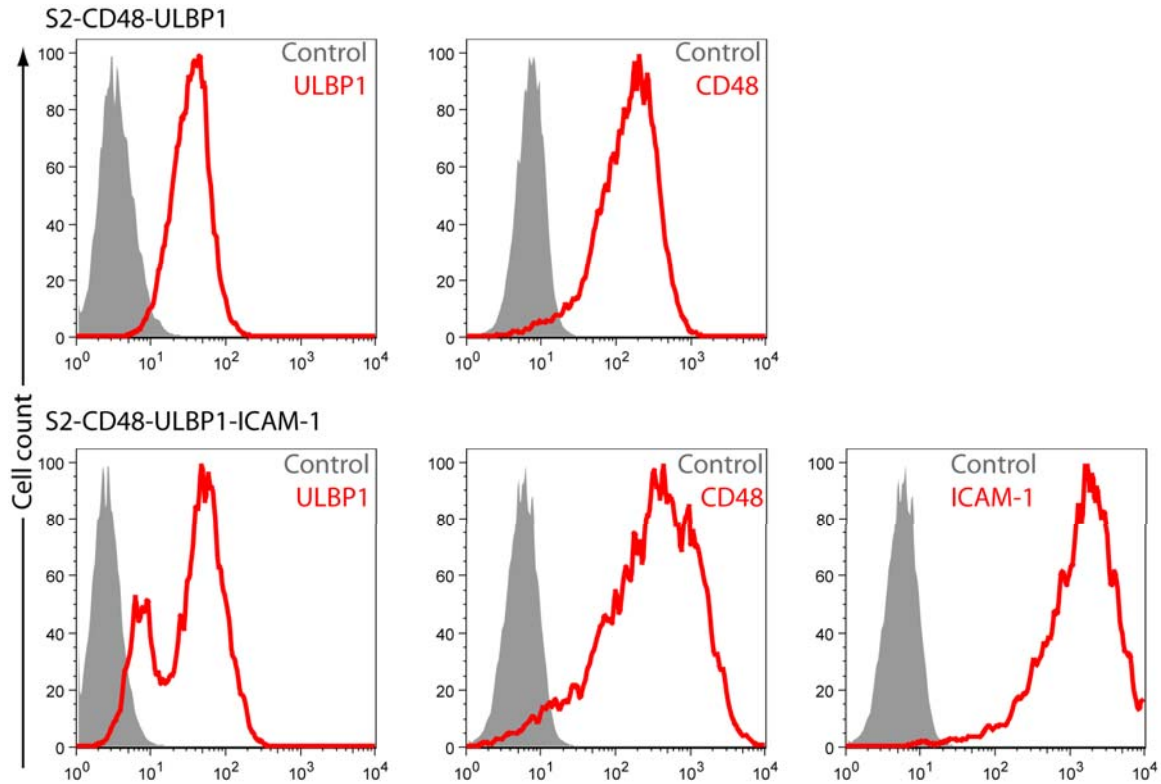


Figure S1: Expression of Human ICAM-1, CD48, and ULBP1 on S2 Insect Cells

S2-CD48-ULBP1 and S2-CD48-ULBP1-ICAM-1 cells were treated with 1 mM CuSO₄ for 48 hr to induce expression of CD48, ULBP1, and ICAM-1. Expression was monitored by staining with Abs to CD48 (clone TU145, BD Biosciences, San Diego, CA), ICAM-1 (clone HA58, BD Biosciences, San Diego, CA), and ULBP1 (clone 170818, R&D systems, Minneapolis, MN).

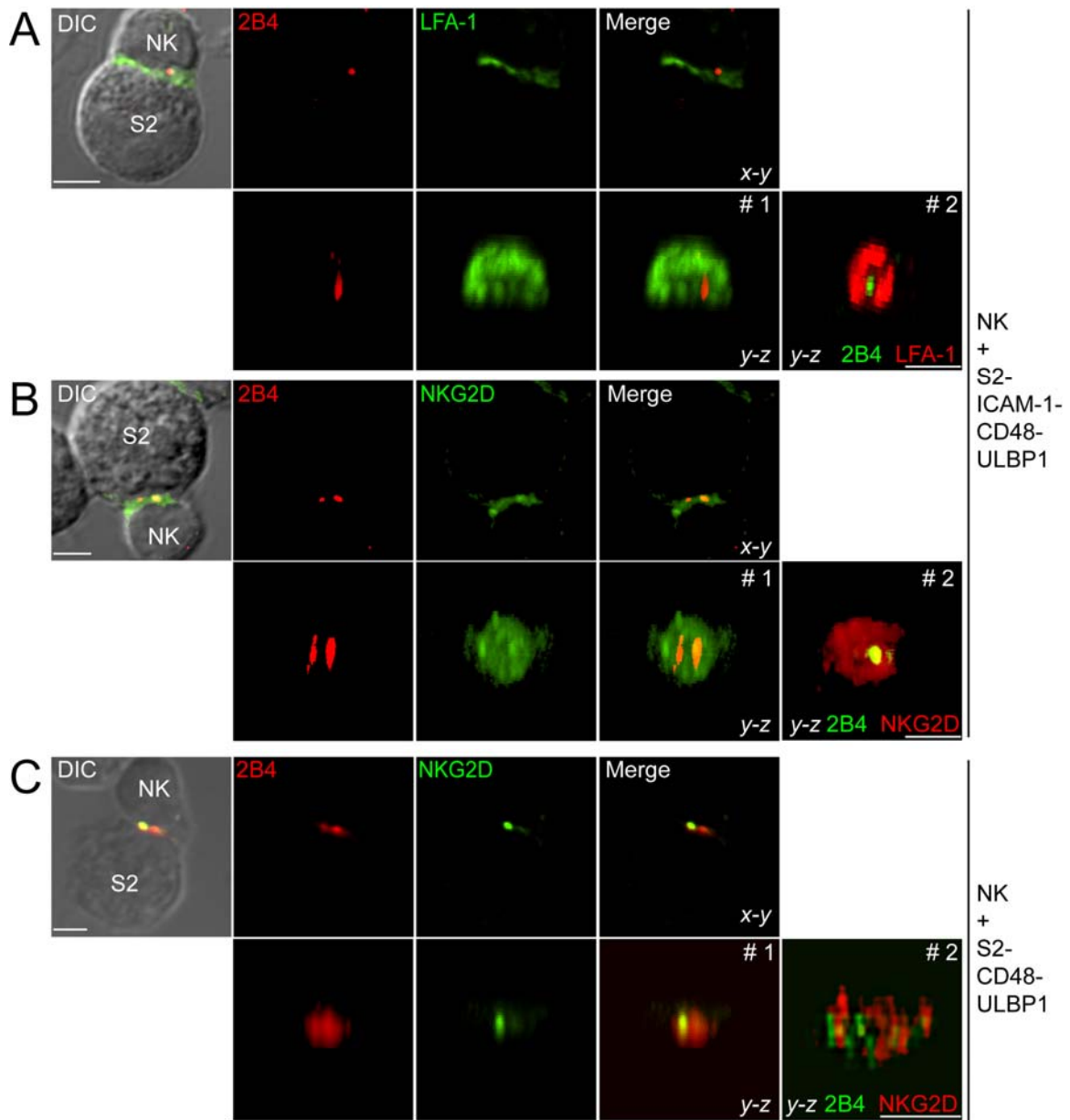


Figure S2: Receptor Distribution in NK Cell Immune Synapses with Transfected Insect Cells

(A) Synapses of NK cells with S2-ICAM-1-CD48-ULBP1 cells stained for 2B4 and LFA-1. NK cells were mixed with S2-ICAM-1-CD48-ULBP1 cells at 37°C for 60 min, fixed, permeabilized, and stained with mAb to 2B4 and LFA-1 followed by Alexa-conjugated secondary antibodies. Top row: Single *x-y* confocal section. Bottom row: *en face* (*y-z*) projections. Cell #2 was stained in reverse colors (2B4 Green, LFA-1 Red). (B) Synapses of NK cells with S2-ICAM-1-CD48-ULBP1 cells stained for 2B4 and NKG2D. NK cells were mixed with S2-ICAM-1-CD48-ULBP1 cells at 37°C for 60 min, fixed, permeabilized, and stained with mAb to 2B4 and NKG2D followed by Alexa-conjugated secondary antibodies. Top row: Single *x-y* confocal section. Bottom row: *en face* (*y-z*) projections. Cell #2 was stained in reverse colors (2B4 Green, NKG2D Red). (C) Synapses of NK cells with S2-CD48-ULBP1 cells stained for 2B4 and NKG2D. NK cells were mixed with S2-CD48-ULBP1 cells at 37°C for 60 min, fixed, permeabilized, and stained with mAb to 2B4 and NKG2D followed by Alexa-conjugated secondary antibodies. Top row: Single *x-y* confocal section. Bottom row: *en face* (*y-z*) projections. Cell #2 was stained in reverse colors (2B4 Green, NKG2D Red). NK cells were fixed and visualized by 3D confocal microscopy. The scale bar is 5.0 μm .

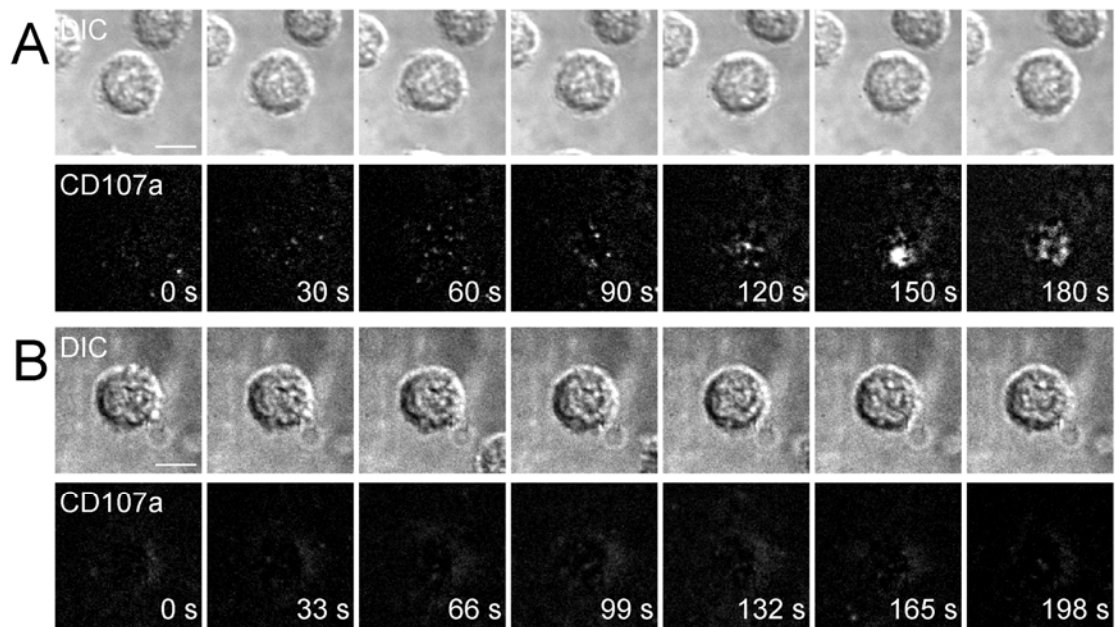


Figure S3. Degranulation Elicited by PMA and Ionomycin

(A) Resting NK cells on lipid bilayers carrying ICAM-1 were stimulated with 100 nM PMA and 10 μM ionomycin and imaged by TIRF microscopy in the presence of 0.016 μM Alexa Fluor 647-conjugated CD107a F(ab). The NK cells were imaged 30 minutes after injection over the lipid bilayer. Movie frames from the indicated time points are shown. Corresponding DIC images are shown on top. Note the dynamic and dispersed distribution of exocytosed LAMP-1. (B) NK cell that was not degranulating imaged 25 minutes after injection over a lipid bilayer carrying IgG1 Fc. Note that 0.016 μM Alexa Fluor 647-conjugated CD107a F(ab) did not label NK cells non-specifically. The scale bar in the first DIC frame is 5.0 μm .

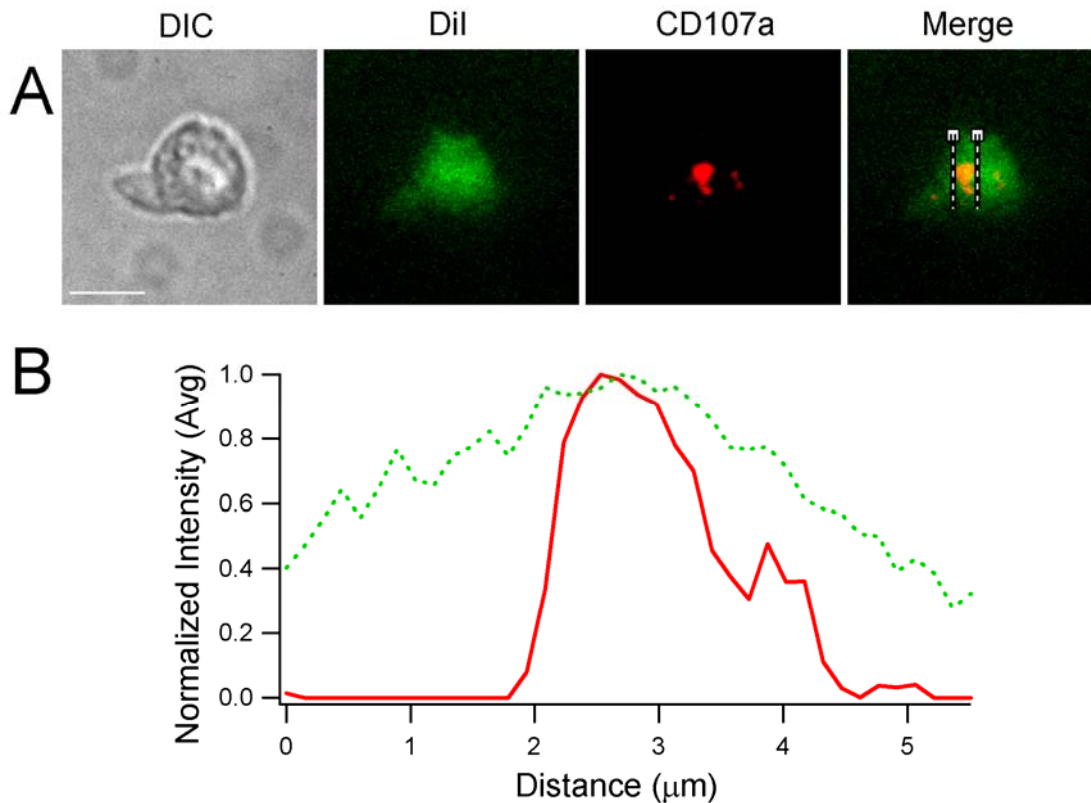


Figure S4. The Central Cluster Detected with CD107a Fab Does not Correspond to Membrane Accumulation

NK cells were loaded with 500 nM DiI16 membrane dye at 37°C for 2 min, and washed twice with PBS. (A) Images were acquired 60 min after injection of NK cells onto lipid bilayers carrying unlabeled Fc and ICAM-1 in the presence of 0.016 μM Alexa Fluor 647-conjugated CD107a F(ab). DiI16 was excited at 514 and measured at 560 ± 25 nm emission. The scale bar in the DIC frame is 5.0 μm. (B) Tracing of the fluorescence intensity of DiI16 (Green) and Alexa Fluor 647 (Red) between the tracks marked in the merged image in (A), normalized to 1 at the peak fluorescence.

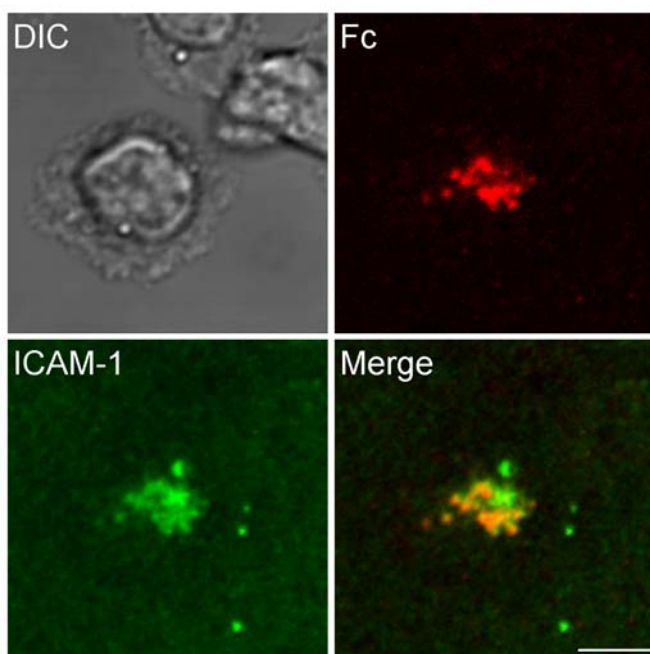


Figure S5. Ligand Distribution in NK Cell Immune Synapse with Lipid Bilayer Containing Fc and ICAM-1

Lipid bilayers containing Alexa Fluor 568-labeled Fc and Alexa Fluor 488-labeled ICAM-1. NK cells were fixed 60 min after injection over lipid bilayer and visualized by confocal microscopy. The scale bar is 5.0 μm .

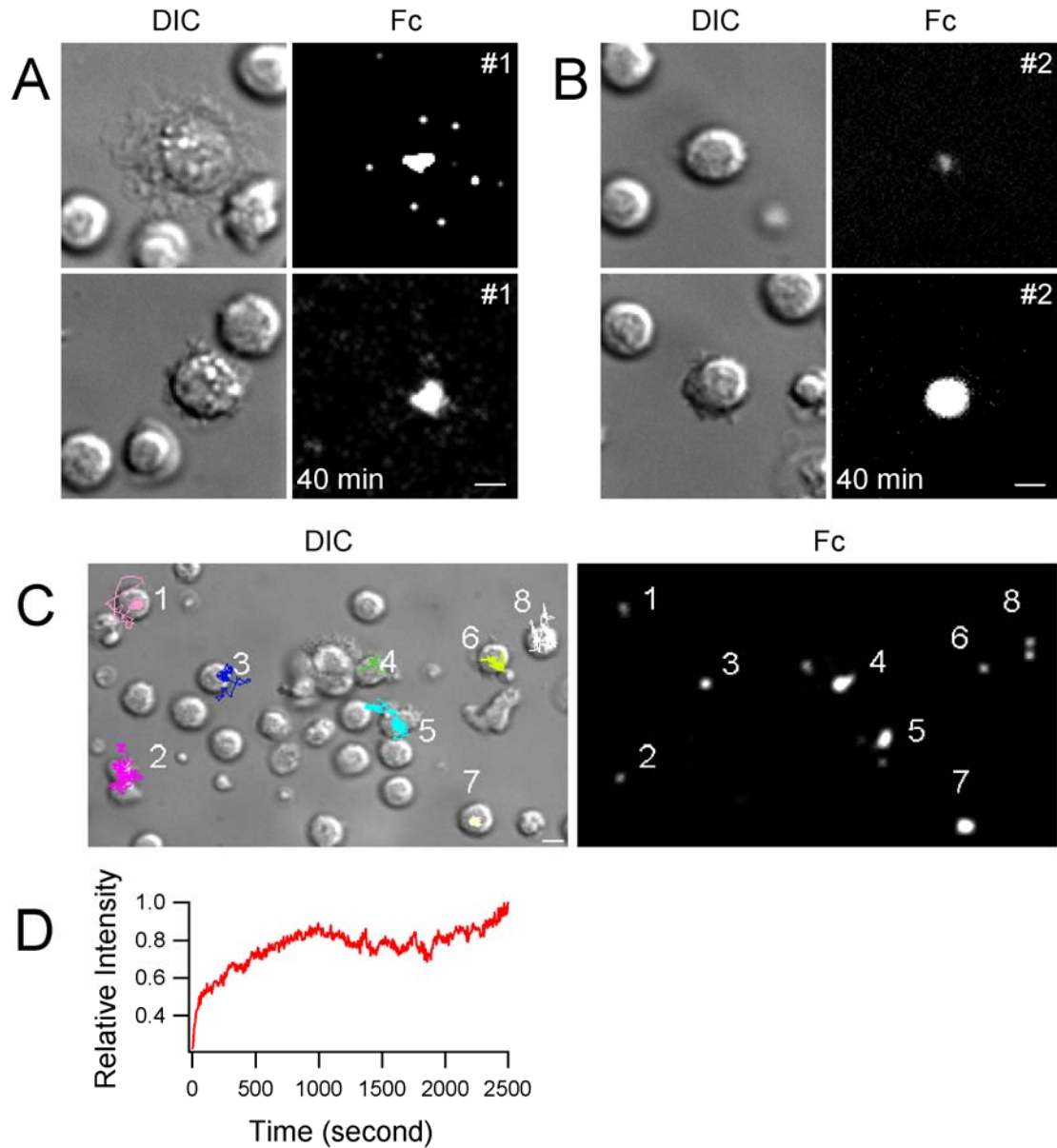


Figure S6. Clustering of IgG1 Fc on Lipid Bilayers with NK Cells

Lipid bilayers carried Alexa Fluor 568-labeled Fc alone. (A) NK cells were visualized 15 minutes after injection over the lipid bilayer. At the beginning, Fc formed several microclusters (top panel). 40 minutes later, Fc accumulated into a single stable cluster

(lower panel). Note in the DIC images that the NK cell was more spread out at the early time point. (B) NK cells were visualized 20 minutes after injection over the lipid bilayer. Over the 40-minute period, the faint Fc signal grew into a bright cluster. (C) Engagement of CD16 alone was also sufficient to arrest the highly motile NK cells. The movement of NK cells and Fc accumulation were tracked over 40 minutes. Eight NK cells that formed contact with the lipid bilayer, as determined by their caged movement (left panel) and by accumulation of Fc visualized by TIRF microscopy (right panel), are numbered. Each colored line represents the recorded path of an individual cell. (D) Relative intensity of Alexa Fluor 568 Fc under cell #7 over time. The scale bar is 5.0 μm . Data are representative of three independent experiments.

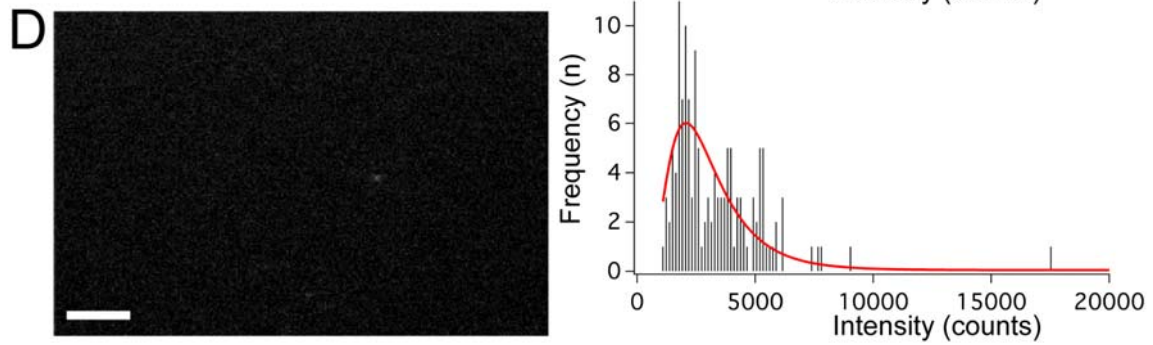
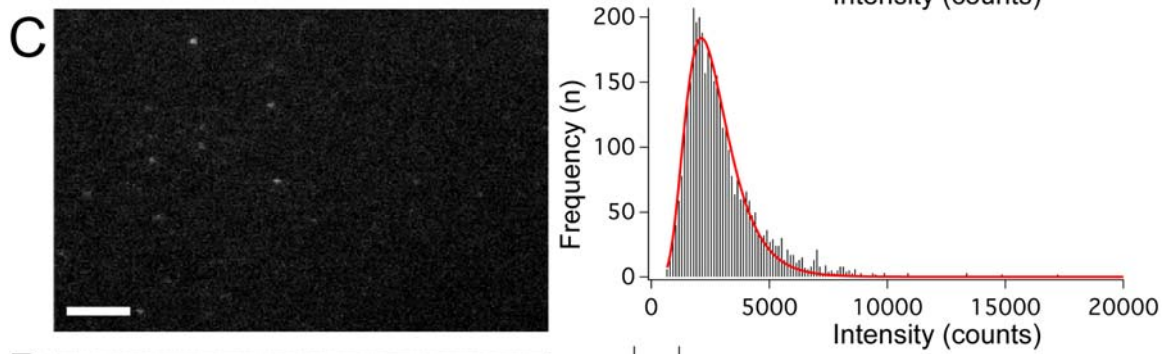
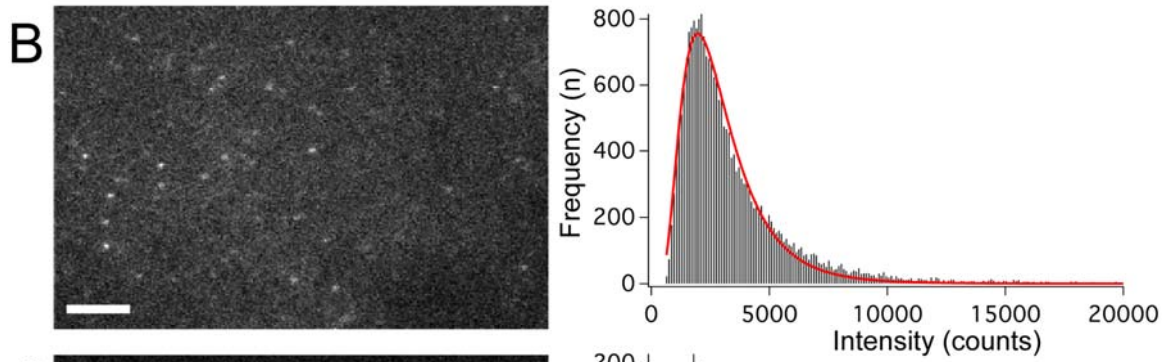
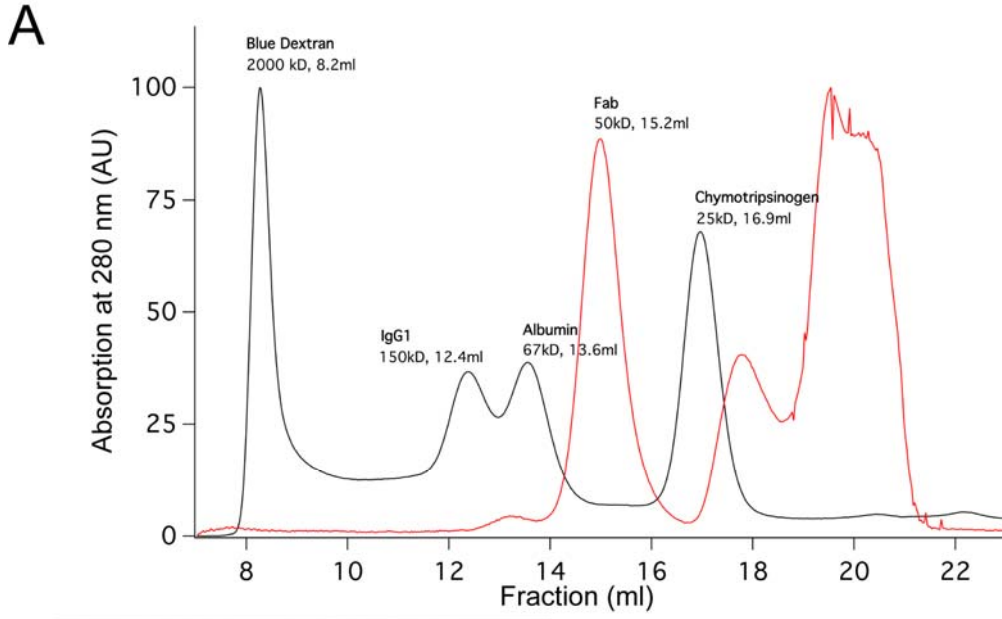


Figure S7. Visualization of Single Fab Molecules

(A) Purification of Alexa Fluor 674-conjugated CD107a Fab by size-exclusion chromatography on Sephacryl S-200. The column was calibrated with the indicated markers. The major peak for the CD107a Fab was found at a position equivalent to 50 kDa, which corresponds to the expected molecular mass of a monomeric Fab. The monomeric Fab carried 0.7 Alexa Fluor dye per molecule. (B, C, and D) Alexa Fluor 674-conjugated CD107a Fab was injected at 160 nM over a lipid bilayer. Fluorescent signals of single Fab molecules binding transiently to the bilayer were imaged by TIRFM. In (C), the chamber was washed with 300 μ l of buffer without Fab and imaged again. In (D), the chamber was washed with 1 ml of buffer without Fab and imaged again. All three images are scaled to the same intensity levels. Intensity distribution of individual fluorescent signals (right panel) showed that peak intensities were independent of the Fab concentration, confirming that single Fab molecules were imaged at all three dilutions. Single-step photo bleaching events further confirmed this result (data not shown). A lognormal fit yielded peaks at intensities of 1979 ± 3 counts ($n = 23809$) in (B), 2127 ± 12 counts ($n = 3844$) in (C), and 2091 ± 95 counts ($n = 147$) in (D). Such a dilution-independent intensity is only possible for single molecules. The fact that discrete single-step photo-bleaching events were observed for the majority of spots (data not shown) is not sufficient evidence that the fluorescent spots represent single Fab molecules, as it could be due to axial movement of the molecule outside of the TIRF field. Scale bars are 5.0 μ m.

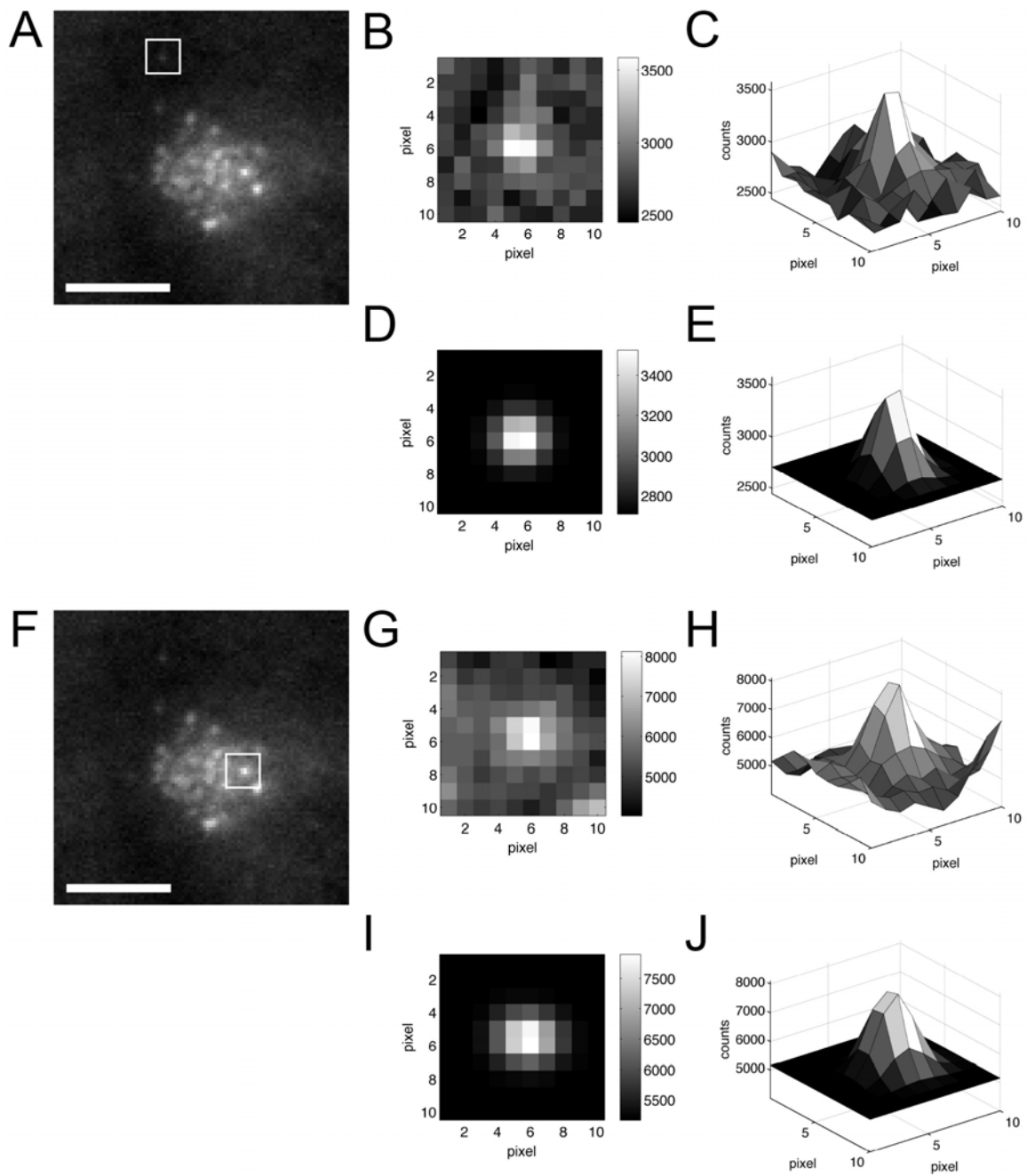


Figure S8. Quantitative Determination of CD107a Fab Fluorescence Intensities

Fluorescence intensities of single Fab molecules and of Fab clusters were determined in the same field as follows. (A to E) Single Fab molecules in areas surrounding the NK cells were selected by an intensity threshold. A sub-image ($1.5 \times 1.5 \mu\text{m}$) centered on each selected spot (white box in A, enlarged in B and C) was fit to a 2-dimensional Gaussian function (D and E). The results of the fit yielded the integrated intensity, background, and full width at half maximum (FWHM). (F to J) Microclusters were manually selected, but subsequently processed as described for single Fab molecule signals. A sub-image was centered on the selected microcluster (white box in F, enlarged in G and H) and fit to a 2-D Gaussian function, which was allowed to adopt elliptical shapes (I and J). Scale bars are $2.0 \mu\text{m}$.

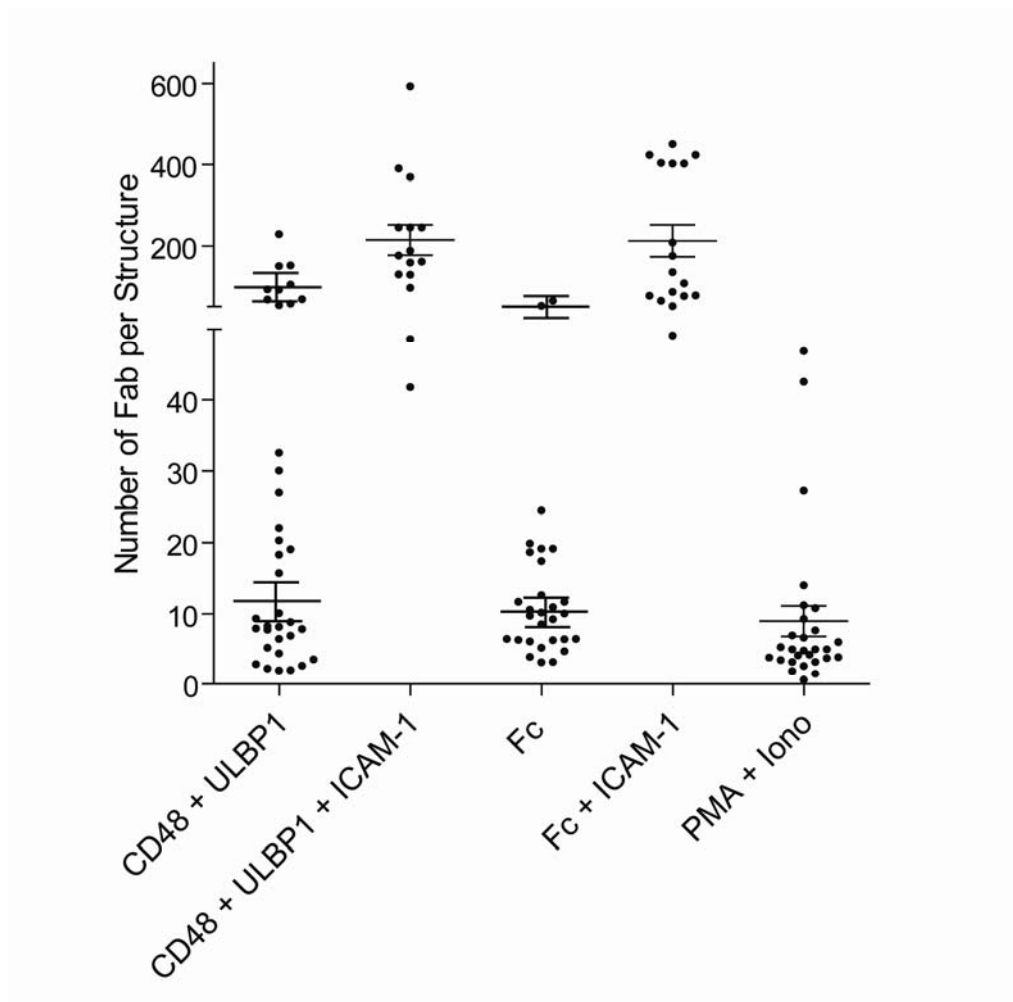


Figure S9. Greater Number of CD107a Fab per Cluster in the Presence of ICAM-1

The number of CD107a Fab per LAMP-1 cluster is shown for different stimulation conditions. NK cells with 16 nM Alexa Fluor 647-conjugated CD107a Fab were injected over lipid bilayers carrying the indicated ligands and imaged about 2 hours later. For stimulation with PMA and ionomycin, NK cells were first injected over bilayers carrying ICAM-1 alone, which does not induce degranulation. The large clusters of LAMP-1 that formed with CD48 and ULBP1 and with Fc alone, were treated as a separate population to determine the average number of Fab molecules. The number of Fab molecules is a

minimal estimate for LAMP-1, as not every LAMP-1 molecule bound an Fab. Bar graphs show mean \pm SEM.

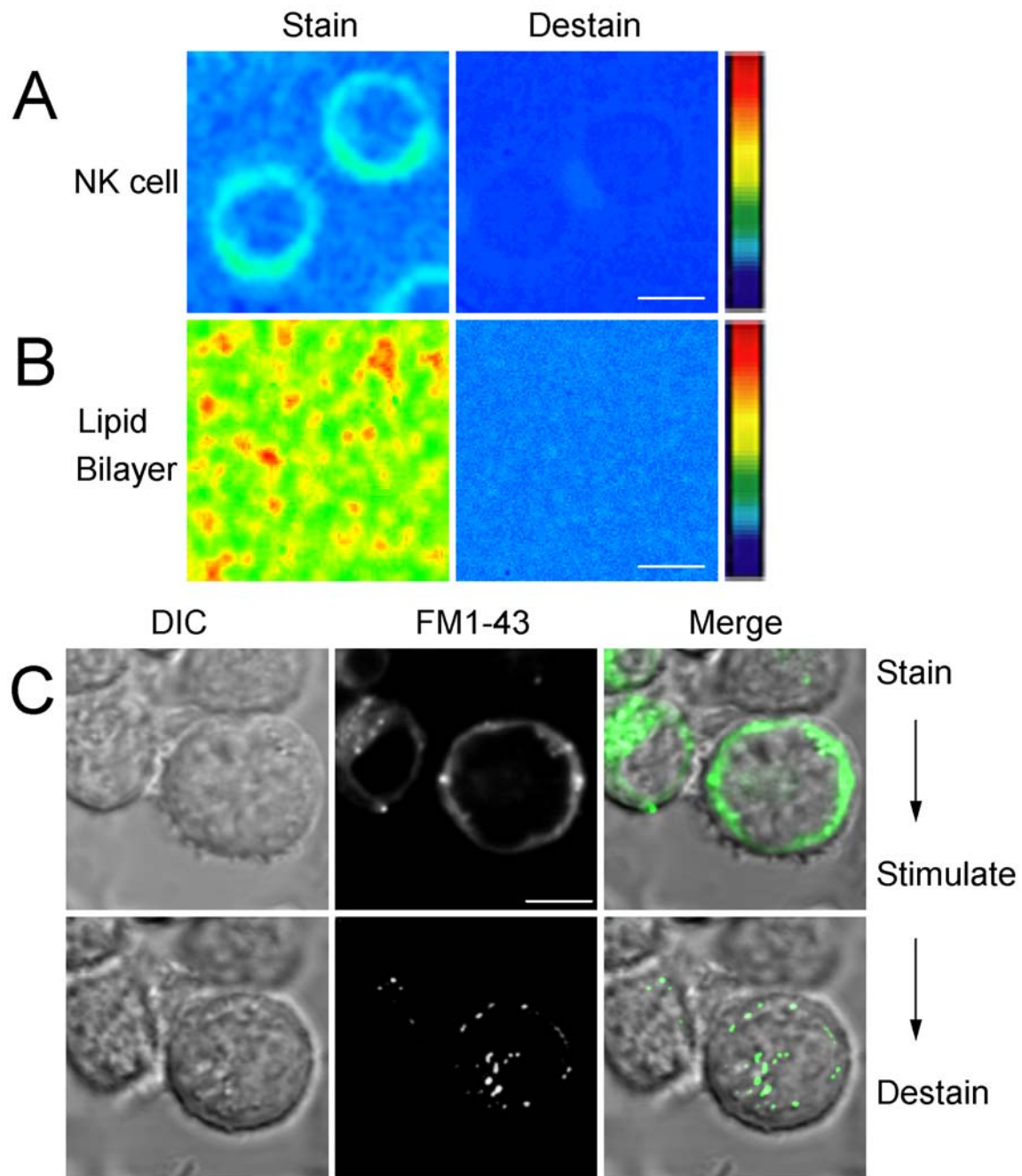


Figure S10. Membrane Internalization Detected by FM1-43 in Live NK Cells

Scale bars are 5.0 μm . The images are representative of 10 cells in three independent experiments. (A) Epifluorescence image of live NK cells stained by FM1-43 within one

min after addition to bilayers carrying unlabeled Fc and ICAM-1 (left) and destained by ADVASEP-7 (right). (B) TIRF image of a lipid bilayer stained by FM1-43 (left) and destained by ADVASEP-7 (right). The FM1-43 intensity is shown as a color-coded scale. (C) DIC and confocal (central panels) images of NK cells stained with FM1-43 and stimulated with PMA and ionomycin for 20 minutes (upper panels). Internalized membrane was visualized after destaining with ADVASEP-7 (lower panels).

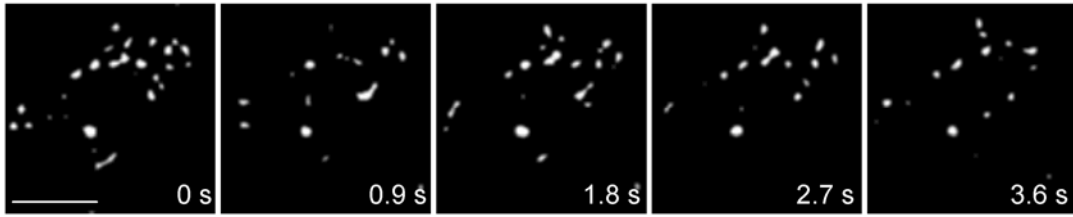


Figure S11. Live Imaging of Lysosomal Compartments

NK cells loaded with 50 nM LysoTracker Green at 37°C for 30 minutes were added onto poly-lysine-coated glass coverslips and imaged by TIRF microscopy. The scale bar is 5.0 μm .

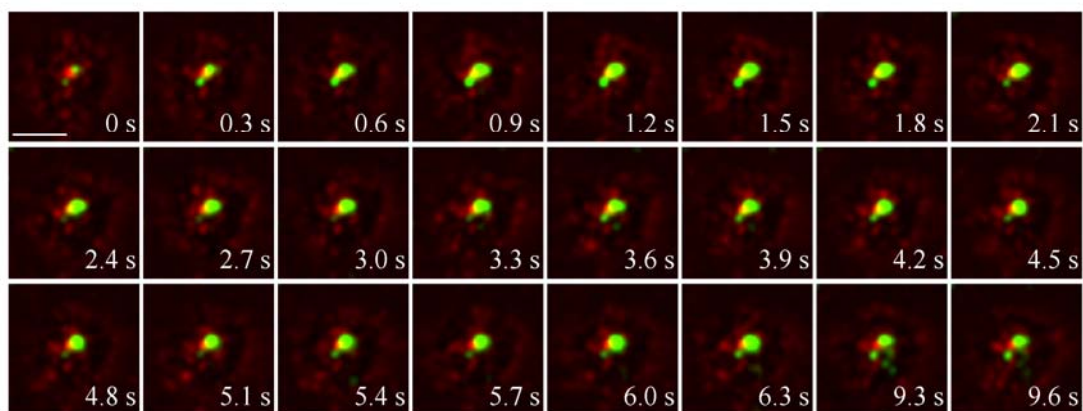


Figure S12. Adjacent Foci of Accumulated LAMP-1 and of Secretory Lysosomes

LysoTracker Green-labeled NK cells were imaged 100 min after injection over a lipid bilayer carrying Fc and ICAM-1 in the presence of 16 nM Alexa Fluor 674-conjugated CD107a Fab. The distribution of lysosomal compartments (Green) and of exocytosed LAMP-1 (Red) was visualized in real time by TIRF microscopy. The scale bar is 5.0 μm . Data are representative of three independent experiments.

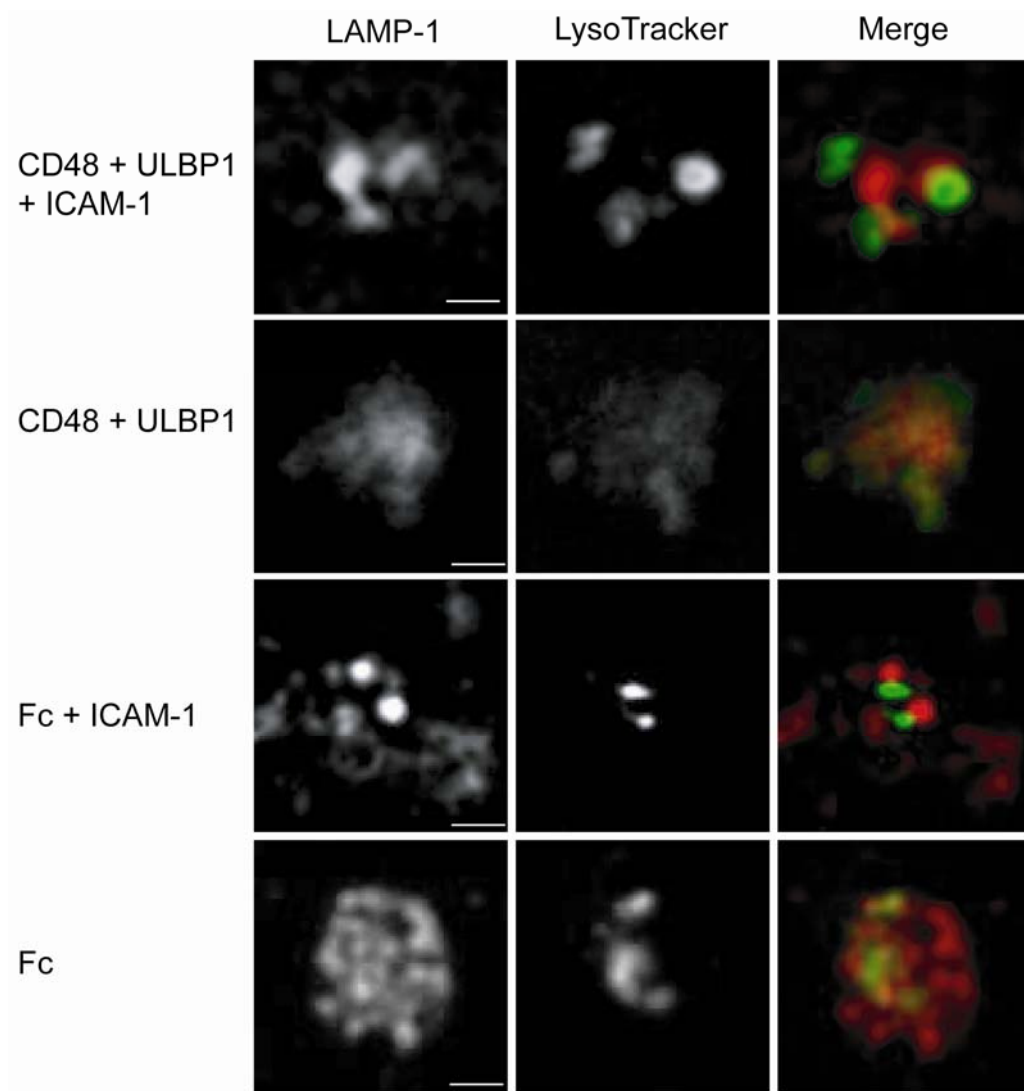


Figure S13. Time Projection of the Lateral Movement of Accumulated LAMP-1 and of Lysosomal Compartments at the Plasma Membrane

NK cells loaded with 50 nM LysoTracker Green were injected over lipid bilayers carrying the indicated ligands in the presence of 16 nM Alexa Fluor 674-conjugated CD107a Fab, and imaged in real time by TIRF microscopy. The 2-dimensional images represent time-projections (*t*-projections), which are the average intensity over time. The projections represent movements over a period of 5 min for bilayer containing

CD48+ULBP1 (\pm ICAM-1) and 2 min for bilayer carrying Fc (\pm ICAM-1), respectively.

The *t*-projections were analyzed in ImageJ. The scale bar is 3.0 μ m.

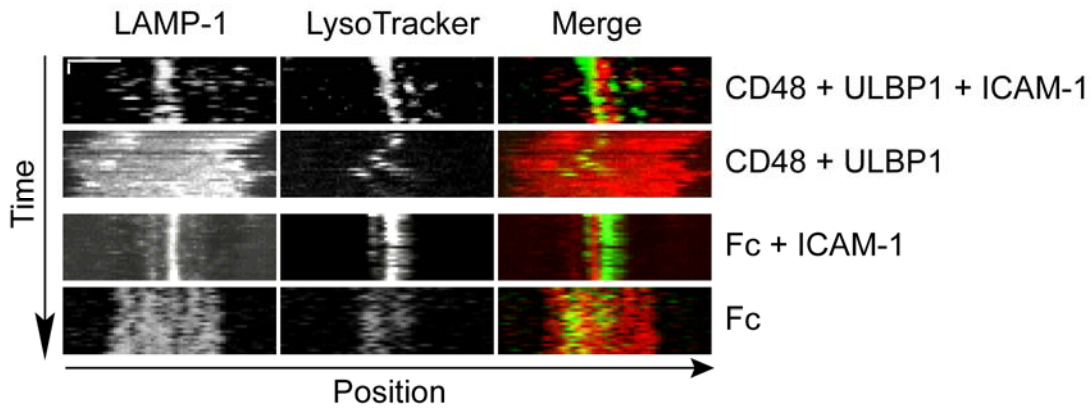


Figure S14. Kymograph of LAMP-1 and Lysosomal Compartments

LysoTracker Green-labeled NK cells were imaged after injection over lipid bilayers carrying the indicated ligands in the presence of 16 nM Alexa Fluor dye conjugated anti-CD107a Fab. Kymographs of LAMP-1 and lysosomal compartments are shown with time increasing from top to bottom (4 min each), and position changes over time on the x - axis. Scale bars represent 3 μm and 40 s.

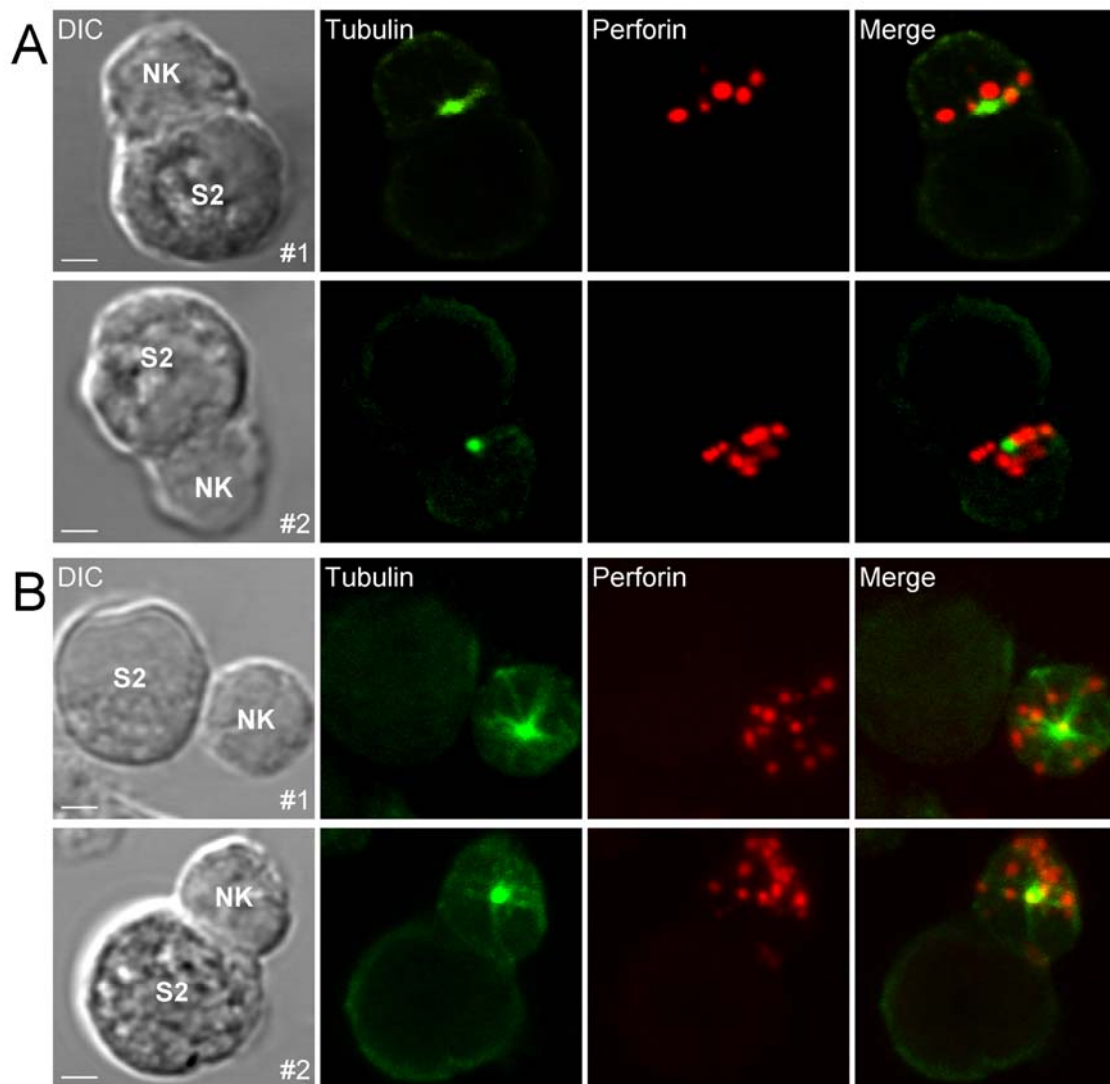


Figure S15. Perforin-Positive Lytic Granules and MTOC Polarize to the Synapse Between NK Cells and S2-ICAM-1 Cells

Scale bars are 2.0 μm. The images are representative of 30 cells in two independent experiments. DIC images are shown on the *Left*. Confocal microscope z-series were obtained. Projected serial confocal sections through conjugation between NK cell and S2

cell are shown. Tubulin was stained Cy3-conjugated anti- β -tubulin (Green). Perforin was stained anti-perforin primary Ab followed by Alexa Fluor 647-conjugated secondary mAb (Red). Merged overlays are on the *Right*. (A) Human resting NK cells were imaged 60 min (#1) and 120 min (#2) after incubation with S2-ICAM-1 cells coated with rabbit IgG. (B) Human resting NK cells were imaged 60 min (#1) and 90 min (#2) after incubation with S2 cells coated with rabbit IgG.

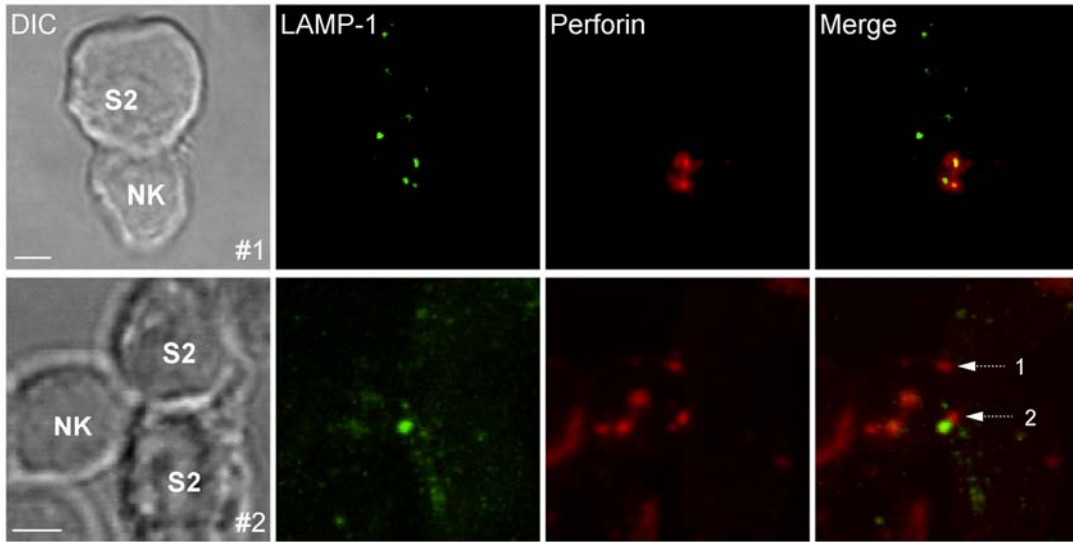


Figure S16. Degranulating NK Cells in Contact with S2-CD48-ULBP1-ICAM-1

Insect Cells

NK cells incubated with transfected S2 cells for 70 min (#1) and 95 min (#2) in the presence of 16 nM Alexa Fluor-555 were fixed, stained for perforin, and imaged by 3-dimensional confocal microscopy. The NK cell in #1 has two LAMP-1 clusters, each one associated with a perforin-containing granule. The lower LAMP-1–perforin pair appears to be inside the cell. The upper one, which appears to be near the plasma membrane, shows some overlap, suggesting the possibility of fusion between the LAMP-1 compartment and cytolytic granules. The NK cell in #2 has formed synapses with two S2 cells, only one of which has resulted in degranulation. A perforin-containing granule (indicated by arrow 1) has polarized towards a synapse in which no degranulation is detected. The other granule (indicated by arrow 2), which is at the cytotoxic synapse, is juxtaposed to the LAMP-1 cluster.

Supplementary Movies Legends

Movies S1, S2, and S3: ICAM-1 Controls the Organization of Natural Cytotoxicity

Immune Synapses

Time-lapsed TIRF images of live NK cells. Scale bars are 5.0 μm . (Movie S1)

Companion to Figure 1D. NK cells were imaged ~60 min after addition to a lipid bilayer carrying ICAM-1-Alexa Fluor 568, ULBP1-Alexa Fluor 488, and unlabeled CD48. Time interval between frames is 10 seconds. (Movie S2) Companion to Figure 1E. NK cells were imaged ~160 min after addition to a bilayer carrying ICAM-1-Alexa Fluor 568, CD48-Alexa Fluor 488, and unlabeled ULBP1. Time interval between frames is 8 seconds. (Movie S3) Companion to Figure 1G. NK cells were imaged ~90 min after addition to a bilayer carrying CD48-Alexa Fluor 568 and ULBP1-Alexa Fluor 488. Time interval between frames is 20 seconds.

Movie S4: Degranulation Elicited by PMA and Ionomycin

Companion to Figure S1A. Time-lapsed TIRF imaging ~15 min after addition of 100 nM PMA and 10 μM ionomycin to live NK cells on bilayers carrying ICAM-1. Time interval between frames is 10 seconds. DIC images (right panel) and Alexa Fluor 647 labeled CD107a Fab (left panel) were displayed simultaneously. The scale bar is 5.0 μm .

Movies S5 and S6: Accumulation of LAMP-1 within a Stable Region at the Center of Cytotoxic Immune Synapses

Movie S5 is a companion to Figure 2B. Time-lapsed TIRF imaging of live NK cells ~120 min after addition to a bilayer carrying CD48, ULBP1, and ICAM-1 (Movie S5) or CD48 and ULBP1 (Movie S6). Time interval between frames is 10 seconds in Movie S5 and 0.33 second in Movie S6. Scale bars are 5.0 μm . CD107a Fab was labeled with Alexa Fluor 647.

Movie S7 and S8: Accumulation of LAMP-1 in a Stable and Central Region at the ADCC Synapse

Scale bars are 5.0 μm . CD107a Fab was labeled with Alexa Fluor 647. (Movie S7) Time-lapsed TIRF imaging of live NK cells ~120 min after NK cell addition to a bilayer containing Fc and ICAM-1. Time interval between frames is 20 seconds. (Movie S8) Companion to Figure 3A, Cell #4. Time-lapsed TIRF imaging of live NK cells ~15 min after NK cell addition to a lipid bilayer containing Fc and ICAM-1. Time interval between frames is 15 seconds. As determined by single-particle tracking, the dispersed LAMP-1 clusters did not move towards a central area.

Movie S9: Early Degranulation Induced by Fc and ICAM-1

Companion to Figure 3B. Time-lapsed TIRF imaging of live NK cells prior to and during degranulation on a bilayer carrying Fc and ICAM-1. Time interval between frames during the first 30 min was 10 seconds. To reduce bleaching, the time interval between frames after 30 min was 30 seconds. Scale bars are 5.0 μm . Images acquired from zero to

32 sec is TIRF fluorescence imaging collected from the lipid bilayer plane prior to injection of Alexa Fluor 647-conjugated Fab and NK cells. The “Anti-CD107a Fab” caption in the movie indicates the time of Alexa Fluor 647-conjugated Fab and NK cell injection into the imaging chamber. The frame at 1 min 46 sec is the first TIRF imaging after including Alexa Fluor 647-conjugated Fab and NK cells into the imaging chamber. The gap between 7 min 8 sec and 16 min 43 sec indicates no specific detectable fluorescence. Three transient degranulation events were detected around 16 min 43 sec, 24 min 35 sec, and 35 min 35 sec. Sustained degranulation was observed around 48 min 14 sec. At that time, a cluster formed at the site that became the prominent and stable cluster. Two minor LAMP-1 clusters were not retrieved into the main cluster.

Movie S10: Central Accumulation of LAMP-1 Despite the Lack of an Organized ADCC Synapse

Time-lapsed TIRF imaging of live NK cells ~110 min after addition to a bilayer carrying Fc and ICAM-1. Time interval between frames is 20 seconds. Scale bars are 5.0 μm . CD107a Fab and Fc were labeled with Alexa Fluor 647 and 488, respectively.

Movie S11: Dynamic Distribution of ICAM-1 and Fc in ADCC Synapses

Time-lapsed TIRF imaging of live NK cells ~20 min after addition to a bilayer carrying Fc-Alexa Fluor 568 and ICAM-1-Alexa Fluor 488. Time interval between frames is 30 seconds. Scale bars are 5.0 μm .

Movies S12, S13, and S14: Dispersed LAMP-1 Micro-Clusters Induced by Fc Alone

(Movie S12) Time-lapsed TIRF imaging of live NK cells ~30 min after addition to a bilayer carrying Fc. Time interval between frames is 0.33 second. The scale bar is 3.0 μm . (Movie S13) Time-lapsed TIRF imaging during early degranulation in live NK cells ~25 min after addition to a bilayer carrying Fc alone. Time interval between frames is 10 seconds. (Movie S14) Time-lapsed TIRF imaging of live NK cells ~37 min after addition to a bilayer carrying Fc alone. Note the formation of large but unstable LAMP-1 clusters. Time interval between frames is 15 seconds. The scale bar is 5.0 μm . CD107a Fab was labeled with Alexa Fluor 647.

Movie S15: Clustering of Fc on the Lipid Bilayer

Companion to Figure S3E and S3F. Time-lapsed TIRF imaging of live NK cells ~5 min after addition to a bilayer carrying Fc-Alexa Fluor 568. (Left panel) NK cell movement tracked in time-lapsed DIC images by the tracking algorithm in Image Pro Plus software. Each colored line represents the path of an individual cell. (Middle panel) Accumulation of Fc-Alexa Fluor 568 on bilayers over time. (Right panel) Fluorescence intensity of Fc over time for the cell highlighted in the first two panels. The time interval between two frames is 5 seconds. The scale bar is 10 μm .

Movies S16 and S17: Active Membrane Internalization at the Site of LAMP-1

Accumulation

Scale bars are 5.0 μm . (Movie S16) Time-lapsed TIRF imaging of live NK cells ~150 min after addition to a lipid bilayer carrying CD48, ULBP1, and ICAM-1. Time interval between frames is 10 seconds. (Movie S17) Time-lapsed TIRF imaging of live NK cells ~120 min after addition to a lipid bilayer carrying Fc and ICAM-1. Time interval between frames is 15 seconds. CD107a Fab was labeled with Alexa Fluor 647. Internalized membrane was detected by FM1-43.

Movie S18: Internalized Membrane and Exocytosed LAMP-1 in the Absence of ICAM-1

Time-lapsed TIRF imaging of live NK cells ~50 min after addition to a bilayer carrying Fc. The scale bar is 5.0 μm . Time interval between frames is 6 seconds. CD107a Fab was labeled with Alexa Fluor 647. Internalized membrane was detected by FM1-43.

Movie S19: Live Imaging of Lysosomal Compartments by TIRF Microscopy

Time-lapsed TIRF imaging of live LysoTracker Green-labeled NK cells ~10 min after addition to a poly-lysine-coated glass coverslip. The scale bar is 5.0 μm . Time interval between frames is 5 seconds.

Movies S20 and S21: Contact of Lysosomal Compartments with the Plasma Membrane at Points Adjacent to Accumulated LAMP-1

Time-lapsed TIRF imaging of live NK cells ~90 min (Movie S20) and ~171 min (Movie S21) after NK cell addition to lipid bilayers containing CD48, ULBP1, and ICAM-1.

Time interval between frames is 10 seconds. Scale bars are 5.0 μm . CD107a Fab was labeled with Alexa Fluor 647. Lysosomal compartments were stained by LysoTrack Green DND-26.

Movies S22 and S23: Lysosomal Compartments and LAMP-1 Microclusters in the Absence of ICAM-1

Time-lapsed TIRF imaging of live NK cells ~88 min (Movie S22) and ~270 min (Movie S23) after NK cell addition to lipid bilayers containing CD48 and ULBP1. Scale bars are 5.0 μm . Time interval between frames is 10 seconds. CD107a Fab was labeled with Alexa Fluor 647. Lysosomal compartments were stained by LysoTrack Green DND-26.

Movies S24 and S25: Lysosomal Compartments Adjacent to Accumulated LAMP-1 during ADCC

Time-lapsed TIRF images of live NK cells on bilayers carrying Fc and ICAM-1. (Movie S24) Companion to Figure 6E. NK cells imaged ~130 min after addition to the bilayer.

The scale bar is 3.0 μm . Time interval between frames is 0.4 second. (Movie S25)

Companion to Figure 6F. NK cells imaged ~50 min after addition to the bilayer. The

scale bar is 5.0 μm . The time interval between two frames is 0.3 second. CD107a Fab was labeled with Alexa Fluor 647. Lysosomal compartments were stained by LysoTrack Green DND-26.

Movie S26: Lysosomal Compartments and LAMP-1 Clusters in the Absence of ICAM-1

Companion to Figure 6G. Time-lapsed TIRF images of live NK cells ~50 min after addition to a bilayer carrying Fc alone. The scale bar is 3.0 μm . Time interval between frames is 10 seconds. CD107a Fab was labeled with Alexa Fluor 647. Lysosomal compartments were stained by LysoTrack Green DND-26.

Movie S27 and S28: 3D Reconstruction of Scanning Confocal Images.

Serial confocal images taken through an NK cell bound to a lipid bilayer carrying Fc and ICAM-1 (Movie S27) and Fc alone (Movie S28). MTOC, perforin, and LAMP-1 are shown in Blue, Green, and Red, respectively. (Movie S27) Perforin-containing lytic granules are directly adjacent to centrally accumulated LAMP-1, and the MTOC is polarized towards the synapse. (Movie S28) Perforin-containing granules and MTOC are not polarized toward the synapse. The signal from LAMP-1 clusters is weak.

Supplemental References

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