Supplementary Information

Perforin pores in the endosomal membrane trigger release of endocytosed granzyme B to the cytosol of target cells

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Supplementary Methods

Cell Lines

HeLa cells were grown to ~60% confluency in DMEM (Gibco) supplemented with 10% FCS, 2 mM glutamine, 2 mM HEPES pH 7.5, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 μM β-mercaptoethanol. YT-Indy, K562 and 721.221 cells were grown in RPMI 1640 (Gibco) supplemented as above.

EEA-1, Lamp-1, GzmB and PFN immunostaining

HeLa cells were grown on rat collagen-coated glass coverslips (Sigma) and treated with sublytic native human PFN and/or GzmB. After the indicated time, cells were fixed for 20 min in PBS with 2% paraformaldehyde (PFA), washed and incubated 20 min in PBS with 50 mM NH₄Cl. Cells were then washed with PBS, permeabilized for 5 min in permeabilization buffer (PBS, 0.2% Triton X-100). After 2 washes in PBS, coverslips were placed in blocking solution (PBS,10% FCS) for 30 min, washed once in PBS and incubated for 1 h at 20°C with the indicated primary antibodies (mouse anti-human PFN, clone Pf80/164, Mabtech Inc.; mouse anti-human PFN, clone Pf344, Mabtech Inc.; goat anti-EEA-1, clone N19, Santa Cruz Biotechnology Inc.; mouse anti-human GzmB, clone GB11, Caltag Laboratories; mouse anti-Lamp-1, clone H4A3, BD Pharmingen) in incubation buffer (PBS, 0.05% Triton X-100). Cells were then washed 3 times with incubation buffer and incubated 1 h at 20°C with donkey
Alexa-Fluor488 and/or Alexa-Fluor647-conjugated secondary antibodies (Molecular Probes) in incubation buffer containing 5% normal donkey serum (Sigma). Cells were then washed 3 times in PBS and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories) before epifluorescence or spinning disk confocal imaging, as indicated. For detection of PFN by flow cytometry, K562 cells were treated at 37 °C with sublytic PFN for the indicated time, fixed for 20 min with PBS in 2% PFA and washed 2 times with PBS in 2% FCS. Half of the cells were stained with the anti-human PFN clone Pf80 and the other half with the anti-human PFN clone Pf344 in PBS with 0.1% saponin before staining with donkey anti-mouse Alexa-Fluor488-conjugated secondary antibody and flow cytometry analysis.

**Inhibition of endosome acidification and intracellular pH measurement**

Inhibition of endosome acidification was performed in HeLa cells by pretreatment with 25 or 50 mM ammonium chloride (Sigma) or Bafilomycin A1 (Sigma) at the indicated concentrations for 60 min. Cells were then washed in HBSS with 10 mM HEPES pH 7.5, 4 mM CaCl₂, 0.4% BSA before PFN-GzmB loading and apoptosis measurement. In some experiments, the same concentration of Ammonium Chloride or Bafilomycin A1 was added during the 2 hr incubation with PFN-GzmB. For endosomal pH measurement, HeLa cells were loaded simultaneously with 10 µg/mL pHrodo 10 KDa dextran (Molecular Probes) and sublytic PFN. For live cell imaging, images were acquired every minute starting 5 min after the addition of dextran ± sublytic PFN in cell loading buffer. The fluorescence intensity of pHrodo dextran within EEA-1⁺ vesicles was analyzed on spinning disk confocal microscopy images using SlideBook 4.2 software. In some experiments, HeLa cells were loaded for 5 min with or without sublytic PFN and the fluorescent pH indicator dye Lysosensor Yellow/Blue DND-160 (Molecular Probes) in cell loading buffer as per the manufacturer’s
instructions. For flow cytometry, PI was added to cells immediately before analysis at a final concentration of 2 μg/mL.

**Epifluorescence and spinning disk confocal microscopy**

Image acquisition by epifluorescence microscopy was performed using a Mariana™ system (Intelligent Imaging Innovations Inc.) consisting of an Axiovert 200M microscope, equipped with a 63x lens (Plan Apochromat, 1.4 NA, Carl Zeiss). Images were analyzed with SlideBook 4.2 (Intelligent Imaging Innovations Inc) to generate three dimensional stacks of optical sections acquired 0.1-0.2 μm apart, before constrained iterative deconvolution using an experimentally determined point spread function (PSF) and maximum Z projection. In some cases, images were acquired by a spinning disk confocal head (Yokogawa) coupled to a fully motorized epifluorescence microscope (Axio Observer) equipped with a 63x lens (Plan Apochromat, 1.4 NA, Carl Zeiss). Two 50 mW solid-state lasers (491 nm and 561 nm; Cobalt Laser) coupled to the spinning head through an acoustic-optical tunable filter (AOTF) were used as light source. The imaging system operates under control of SlideBook 4.2 and includes a computer controlled spherical aberration correction device (SAC, Intelligent Imaging Innovations, Inc) installed between the objective lens and the back illuminated CCD camera (Cascade 512B; Roper Scientific, Photometrics). To reduce photo-bleaching the illumination was turned off during the readout period from the CCD to the computer. Typically, time-lapse images were acquired with exposure times between 100 and 300 msec. For immunostaining, secondary antibody alone or Ig control were included to establish specificity of antibody staining (not shown). For co-immunostaining, Alexa488 and Alexa647-conjugated secondary antibodies were used to avoid bleed-through. For live cell imaging, EGFP or mRFP-fusion protein-transfected cells were imaged first independently in both channels to set the exposure time and avoid bleed-through.
**Apoptosis measurement**

To assess apoptosis, cells incubated for 2 h at 37°C with buffer or sublytic rat PFN and/or 200 nM native human GzmB were analyzed for caspase activation by flow cytometry using M30-FITC mAb\(^{50}\) staining according to the manufacturer’s protocol (M30 CytoDEATH, Roche) to detect a caspase-cleavage product of cytokeratin 18. For apoptosis assays by AnnexinV and PI staining, cells were incubated 45 min at 37°C with buffer or sublytic rat PFN and/or 100 nM native human GzmB. Cells were then washed in annexin buffer (10 mM HEPES pH7.4, 140 mM NaCl, 2.5 mM CaCl\(_2\)), incubated 10 min with Annexin-V-APC (Caltag), washed once, resuspended in annexin buffer containing 2 µg/ml PI and analyzed by flow cytometry. Caspase-3 activation was tested by immunoblot (anti-caspase-3, clone 3G2, Cell Signaling; anti-actin, Sigma-Aldrich) 30 min after treatment with buffer or sublytic rat PFN and/or 50 nM native human GzmB.

**Chromium Release Assay**

Cytotoxicity was measured by 4 h chromium release assay. Briefly, \(^{51}\)Cr-labeled 721.221, pre-incubated for 2 h with 200 nM bafilomycin A1, were washed and incubated for 4 h at 37°C in bafilomycin A1-free medium with effector cells (YT-Indy) at different effector/target ratios in a final volume of 200 µL in 96-well microplates. Experiments were performed in triplicate. At the end of the incubation 50 µL of the supernatant was transferred into 96-well LumaPlate solid scintillation plates (Packard Instrument Co.) and counted in a Top Count counter (Packard) after overnight drying. Data were expressed as the percentage of specific \(^{51}\)Cr release from target cells, calculated as (experimental release-spontaneous release)/(maximum release-spontaneous release) x 100.
**Fusion assay**

Cells transfected to express a transferrin receptor-eGFP fusion protein were incubated for 1 hr with Cy5-conjugated anionic dextran sulfate, washed and then treated for 5 min with buffer or sublytic PFN before analysis by spinning disk confocal microscopy.

**Quantification of endosome/gigantosome size**

The size of endosomes-gigantosomes was analyzed on confocal microscopy pictures using SlideBook 4.2 software by measuring the surface area of each mRFP-EEA-1⁺ endosome in cells treated with buffer or sublytic PFN. At least 2 buffer or 3 PFN-treated cells were analyzed.

**Statistical analysis**

Data are expressed as mean ± standard deviation (s.d.). P values were determined by unpaired two-tailed student’s t-test.

**Supplementary reference**

50. Leers, M.P. *et al.* Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* **187**, 567-572 (1999).
Supplementary Figure 1. PFN-induced gigantosomes form by homotypic fusion of early endosomes

(a) Within 3 min of treatment with sublytic native rat PFN, large intracellular and membrane bound endosomes (gigantosomes) form in EGFP-EEA1 transfected HeLa cells. Confocal pictures are representative of six independent experiments. Percentage of cells with enlarged endosomes is indicated (mean ± s.d.).

(b) Gigantosomes stain for EEA-1, but not Lamp-1, 10 min after treatment with rat PFN. DAPI was used to visualize nuclei. Widefield images are

Supplementary Figures
representative of five independent experiments. Percentage of cells with EEA-1+ Lamp-1- gigantosomes is indicated (mean ± s.d.) (c-d) Large endosomes form by fusion of early endosomes. EGFP-EEA1 transfected HeLa cells, treated or not with sublytic rat PFN, were imaged by spinning disk confocal microscopy. Within 2 min of PFN treatment, EEA-1+ early endosomes start to fuse together to form gigantosomes. Gigantosome size increases by multiple sequential fusion events. Data are representative of five independent experiments. (e) Gigantosomes form by fusion with previously formed endosomes. Cells transfected to express an EGFP-transferrin receptor fusion protein (EGFP-TFR) were loaded for 1 h with Cy5-conjugated anionic dextran, washed and then treated for 5 min with medium or sublytic human PFN. Some of the cell surface receptor and pinocytosed fluid phase dextran become incorporated into gigantosomes in the presence of PFN. Percentage of cells with EGFP-TFR+dextran+ gigantosomes is indicated (mean ± s.d.). Color bars and associated numbers indicate fluorescence intensity levels. Scale bars, 10 µm. Dashed lines, plasma membrane.
Supplementary Figure 2. PFN-induced gigantosome formation requires Rab5

(a) Expression of dominant negative Rab5(S34N) mutant inhibits gigantosome formation. HeLa cells were transiently transfected with mRFP-EEA1 alone or together with EGFP-Rab5(WT), EGFP-Rab5(S34N) or constitutively active EGFP-Rab5(Q79L). Cells were imaged by live cell spinning disk confocal microscopy 10 min after sublytic rat PFN treatment. Gigantosomes form in cells expressing EEA1 and the wild-type form of Rab5, while the dominant-negative Rab5(S34N) mutant inhibits gigantosome formation. Co-

b

- PFN
+ PFN (10 min)

- PFN
+ PFN (10 min)

- PFN
+ PFN (10 min)

- PFN
+ PFN (10 min)
transfection of HeLa cells with EGFP-Rab5(Q79L) and mRFP-EEA1 results in enlarged endosomes in the absence of PFN which become even larger after treatment with sublytic rat PFN. Pictures are representative of three independent experiments. Percentage of cells with gigantosomes is indicated (mean ± s.d.). Color bars and associated numbers indicate fluorescence intensity levels. Scale bars, 10 µm. Dashed lines, plasma membrane. (b) Area occupied by EEA-1+ endosomes in confocal sections of cells transfected as in (a) and treated with buffer or sublytic rat PFN for 10 min. Each symbol represents one endosome and the bars represent mean area. P values were determined by unpaired two-tailed student’s t-test (NS: not significant).
Supplementary Figure 3. Inhibition of Rab5-dependent homotypic fusion does not inhibit GzmB-induced apoptosis

HeLa cells transfected with EGFP-Rab5(WT) or EGFP-Rab5(S34N) (to obtain about 50% of EGFP+ cells) were treated with buffer or sublytic rat PFN ± 100 nM native human GzmB. Apoptosis of EGFP− and EGFP+ cells from the same sample was measured 45 min later by Annexin V/PI staining. Mean ± s.d. from three independent experiments (AnnexinV+ PI−/− cells) are shown. P values were determined by unpaired two-tailed student’s t-test. No significant (NS) difference was observed in GzmB-mediated apoptosis in Rab5(S34N)-transfected cells relative to Rab5(WT)-transfected cells or between transfected and untransfected cells in the same sample.
Supplementary Figure 4. Endosomal acidification is inhibited by PFN and blocking endosomal acidification does not affect PFN-mediated cell death

(a) HeLa cells, treated simultaneously with sublytic human PFN, Lysosensor Yellow/Blue and PI, lose Lysosensor green fluorescence within 5 min, but maintain membrane integrity (do not incorporate PI). These data indicate that PFN disrupts organelle acidification, without causing cell death. (b) Inhibition of endosome acidification does not alter PFN-induced cell death. HeLa cells, pre-incubated with the indicated dose of bafilomycin A1 for 1 h to inhibit endosome acidification, were treated with sublytic rat PFN. Cell death was quantified 20 min later by PI staining. Mean ± s.d. from two independent experiments are shown. P values were determined by unpaired two-tailed student’s t-test. No significant (NS) difference was observed between bafilomycin A1 versus DMSO or buffer treated cells.
Supplementary Figure 5. PFN staining suggests PFN-pore formation in the endosomal membrane

(a) Single confocal section and z-stack series of one gigantosome stained with anti-PFN (Pf80) (black) 7 min after HeLa cell treatment with sublytic human PFN. (b) Representative confocal z-stack series of PFN staining (Pf80) (pseudocolor) in a gigantosome. Images are representative of six independent experiments. (c) Z-stack spinning disk confocal optical sections of gigantosomes. Images were acquired 0.1 μm apart after staining with anti-PFN.
(Pf344) 1, 7 or 15 min after HeLa cells were treated with sublytic human PFN. Pictures are representative of three independent experiments. Color bars and associated numbers indicate fluorescence intensity levels. Scale bars (a,b) 5 µm; (c) 2 µm. Dashed lines, plasma membrane. (d) Detection of PFN aggregates after crosslinking with DSS. Target cells were incubated with sublytic native human PFN for the indicated time before isolation of the vesicular fraction. The cell-permeable cross-linking agent DSS was added to the fractionated vesicles. PFN immunoblot shows PFN monomer (60 kDa) as well as a PFN multimer of ~420 kDa and a larger multimer migrating near the top of the gel after 10 min. Data are representative of two independent experiments.
Supplementary Figure 6. Endocytosed dextran is released from gigantosomes into the cytosol
(a,b) Representative time lapse series of gigantosomes imaged in live cells 9-17 min after EGFP-EEA-1-transfected HeLa cells were incubated with 10 kDa cationic TR-dextran and sublytic rat PFN. Scale bars, 2 µm. The movies from which these images were extracted are provided in Supplementary movies 2-3. Data are representative of three independent experiments.
Supplementary Figure 7. Model for PFN delivery of Gzms.

After cytotoxic granule exocytosis into the immunological synapse (1), PFN multimerizes in the target-cell membrane to form small pores through which Ca\(^{2+}\) enters (2), triggering a plasma membrane repair response (3) in which lysosomes fuse with the damaged plasma membrane and PFN and Gzms are rapidly internalized by dynamin and clathrin-dependent endocytosis. PFN and Gzm-containing endosomes then fuse together by rapid Rab5-dependent homotypic fusion in response to the transient Ca\(^{2+}\) flux (4) to form gigantosomes. Within gigantosomes, PFN continues to multimerize to form new and possibly bigger pores, preventing acidification and causing some Gzm release (5), before inducing endosomal rupture and complete Gzm release into the target-cell cytoplasm (6), where they initiate programmed cell death.
Supplementary Movie 1. PFN-mediated release of endocytosed dextran from a gigantosome

Representative EGFP-EEA-1+ (green) gigantosomes from transfected HeLa cells treated with TR-Dextran (red) and sublytic rat PFN. Live images were acquired by spinning disk confocal microscopy starting 10 min after addition of TR-Dextran and PFN (duration 5 min, 2.5 sec/frame). Selected static individual frames from these movies are shown in Fig. 5c.

Supplementary Movie 2. PFN-mediated release of endocytosed dextran from a gigantosome

Representative EGFP-EEA-1+ (green) gigantosomes from transfected HeLa cells treated with TR-Dextran (red) and sublytic rat PFN. Live images were acquired by spinning disk confocal microscopy starting 10 min after addition of TR-Dextran and PFN (duration 6 min, 10 sec/frame). Selected static individual frames from these movies are shown in supplementary Fig. 6a.

Supplementary Movie 3. PFN-mediated release of endocytosed dextran from a gigantosome

Representative EGFP-EEA-1+ (green) gigantosomes from transfected HeLa cells treated with TR-Dextran (red) and sublytic rat PFN. Live images were acquired by spinning disk confocal microscopy starting 10 min after addition of TR-Dextran and PFN (duration 13.5 min, 10 sec/frame). Selected static individual frames from these movies are shown in supplementary Fig. 6b.