Supporting Information

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SI Materials and Methods

Cell Culture, Treatment, and Transfection. Human MCF-7 and T47D breast cancer cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 supplemented with 10% (vol/vol) FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Lonza). MCF–7beclin1 (BECN1) tetracycline-responsive promoter (tet-off) cells, which were kindly provided by B. Levine (University of Texas Southwest Medical Center, Dallas), were maintained in RPMI-1640 supplemented with 200 μg/mL hygromycin and 100 ng/mL doxycycline (1).

Cell transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. MCF-7 cells were stably transfected with a GFP–LC3–encoding vector containing a geneticin resistance gene for selection (kindly provided by N. Mizushima, Tokyo Medical and Dental University, Tokyo). ATG5-defective MCF-7 cells (ATG5−) were generated by stable infection with ATG5 shRNA lentiviral particles (Santa Cruz Biotechnology) containing a puromycin resistance gene for selection. Control MCF-7 cells (ATG5+) were infected with scrambled shRNA. Targeting p62/SQSTM1 was performed by transfection of cells with p62 siRNA (Qiagen). Unless otherwise indicated, hypoxia was generated by incubating cells in 0.1% (vol/vol) pO2 for 16 h. Alternatively, autophagy was induced by cultivating cells in Earle's Balanced Salt Solution (EBSS) for 16 h. Lysosomal hydrolases were inhibited by treatment of cells with e64d/pepstatin (200 ng/mL) for 16 h. Inhibition of autophagic flux was performed by incubating cells with chloroquine (60 μM) for 1 h. YT–Indy–natural killer (NK) cells, expressing eGFP–granzyme B, were used for specific experiments and were grown as previously described (2). Murine melanoma B16–F10 cells were obtained from the ATCC and grown in DMEM supplemented with 10% (vol/vol) FCS. YT–Indy–NK cells, expressing eGFP–granzyme B, were used for specific experiments and were grown as previously described (2). Murine mammary carcinoma 4T1 cells were obtained from the ATCC and grown in RPMI-1640 supplemented with 10% (vol/vol) FCS. Cells were stably infected with either scrambled or Beclin1 shRNA lentiviral particles (Santa Cruz Biotechnology) containing a puromycin resistance gene for selection.

NK Cell Isolation. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors. An NK Cell Isolation Kit (Miltenyi Biotec) was used to isolate NK cells from the PBMC according to the manufacturer's instructions. The purity and viability of isolated NK cells were evaluated by flow cytometry (≥98% and 98%, respectively). NK cells were activated for 20 h in RPMI-1640 medium supplemented with 300 U/mL of human recombinant Interleukin-2 (R&D Systems).

Subcellular Fractionation. Cells were harvested and homogenized in buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM acetic acid, 10 mM triethanolamine, 20 mM *N*-ethylmaleimide, and 2 mM PMSF, pH 7.4. Cells were disrupted by passing them through a 25G needle five times, and a clear homogenate was generated by two consecutive centrifugations at 1,000 × *g* for 5 min at 4 °C. The supernatant (500 μL) was loaded onto the top of a discontinuous OptiPrep (Axis-Shield) gradient [0.75 mL each of 30, 26, 22, 18, 14, and 10% (vol/vol) solutions] and submitted to ultracentrifugation at 268,000 × *g* for 310 min in a MLS-50 rotor (k factor = 71, Optima MAX-XP centrifuge, Beckman). Twelve fractions (400 μL) were collected from the top of the gradient, lysed in 1× Laemmli buffer, and analyzed by immunoblot.

Granzyme B Loading. Cells (1.5×10^5 cells) were loaded with exogenous granzyme B (GzmB) from human lymphocytes (Enzo Life Sciences) using sublytic doses of preactivated pore-forming protein streptolysin-O (SLO) (Sigma). Cells were washed in Hank's Balanced Salt Solution (HBSS), followed by the addition of 100 μL of HBSS with 0.5% (vol/vol) FBS and 100 nM of both GzmB and SLO. The cells were incubated for 2 h at 37 °C before analysis using multicolor flow cytometry (FACSCanto). Results are reported as a percentage of death for normoxic (N) and hypoxic (H) cells and normalized to cells treated with SLO alone.

Flow Cytometry Analysis. Cells were stained for 30 min at 4 °C with the following antibodies: HLA–ABC–phycoerythrin (PE) and NK1.1–PE/Cy7 (BioLegend); HLA–E–PE and HLA–G–APC (eBiosciences); ULBP-1–PE, MIC–A/B–PE, and MIC–A–APC (R&D Systems); CD56–PE/Cy7 and CD3–FITC (ImmunoTools); and CD107a–APC and CD49b–APC (BD Biosciences). Surface expression levels of each protein were assessed using flow cytometry (FACSCanto).

Cell Lysates and Immunoblot. Cells were lysed in RIPA Buffer (Sigma Aldrich) supplemented with a protease and phosphatase inhibitor mixture (Roche). Lysates were centrifuged at 15,000 × *g* for 20 min at 4 °C, and supernatants were collected. Protein concentration was determined using a BioRad protein assay kit. Proteins were separated by SDS/PAGE, transferred onto PVDF membranes, and blocked with 5% (vol/vol) skim milk in 0.1% (vol/vol) Tween 20 in TBS. The following primary antibodies were used: HIF-1α and p62 (BD Biosciences); LC3B, Beclin1, granzyme B, Atg5, Smac, Calnexin, heat shock protein (HSP) 70, and Histone H3 (Cell Signaling); actin (Sigma Aldrich); perforin (Biolegend); EEA1 Rab5A (Santa Cruz Biotechnology); GFP (Chemicon); and M6PR (Abcam). After incubation with primary antibodies, immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research Laboratories). Protein bands were revealed by enhanced chemiluminescence ECL (GE Healthcare).

Time-Lapse Live Video Microscopy and Confocal Imaging. Cells were precultured under normoxic or hypoxic conditions for 24 h. Isolated NK cells were preactivated as described above and stained with PKH-26 (red) (Invitrogen) according to the manufacturer's recommendations. GFP–LC3–expressing normoxic or hypoxic MCF-7 cells were incubated with labeled NK cells at a 5/1 E/T ratio in μ-Slide eight-well chambered coverslips (IBIDI) and analyzed with a Zeiss LSM-510 Meta laser scanning confocal microscope (Carl Zeiss). In specific time-lapse experiments, propidium iodide (1.5 μM) was added to the cell culture medium as a cell death indicator. Cells were maintained at 37 °C in a CO_2 incubator mounted on the microscope stage. Time-lapse microscopy was performed using an Axiovert 200M microscope (Carl Zeiss MicroImaging), and images were captured with a 40× oil objective lens at intervals of one frame per 1–2 min during 82 min for normoxic cells and 86 min for hypoxic cells.

For specific experiments, autophagosomes and endosomes were visualized using an LSM-510-Meta confocal microscope (Carl Zeiss). These cellular structures were detected after staining with anti-LC3 (Cell Signaling) and anti-EEA1 (Santa Cruz Biotechnology) primary antibodies, respectively, followed by incubation with Alexa-Fluor-488/568–coupled secondary antibodies. DAPI was used to stain nuclei (blue).

Cell Doubling-Time Evaluation. Cell doubling was evaluated using a colorimetric cell viability assay (XTT). Briefly, cells were seeded in 96-well plates, and cell viability was determined every 24 h for 3 d by measuring absorbance at 450 and 630 nm after the addition of 50 μL of 1 mg/mL XTT solution (Sigma Aldrich). Growth curves were plotted between 24 and 72 h, and the cell-doubling time was calculated.

Formation of Conjugates. NK cells isolated from healthy donors were stained with PKH-26 (red), and MCF-7 cells were stained with CellTrace CFSE (green) according to the manufacturer's instructions. Effector and target cells (E/T ratio: 5/1) were co-cultured at 37 °C for 0, 5, 10, and 20 min. At each time point, the samples were immediately fixed in ice-cold 0.5% (vol/vol) paraformaldehyde. The double-positive population (E/T conjugates) was assessed using flow cytometry (FACSAria).

Degranulation Assay. Purified and activated NK cells were incubated for 5 h alone or with target tumor cells at a 5/1 E/T ratio in the presence of anti-human CD107a antibody (BD Biosciences). Golgi Stop (BD Biosciences) was added after 1 h of incubation to block intracellular protein transport processes. The NK cells were stained for 30 min with anti-CD56 antibody (ImmunoTools) and LIVE/DEAD dye (Invitrogen) according to the manufacturer's recommendations. NK cells incubated without target cells for 5 h were used as a negative control. Samples were analyzed using flow cytometry (FACSCanto).

Statistical Analysis. Statistical analyses were performed using a two-tailed Student *t* test. Differences were considered statistically significant when the *P* value was <0.05.

1. Liang XH, Yu J, Brown K, Levine B (2001) Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. *Cancer Res* 61(8):3443–3449.

2. Thiery J, et al. (2011) Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol* 12(8):770–777.

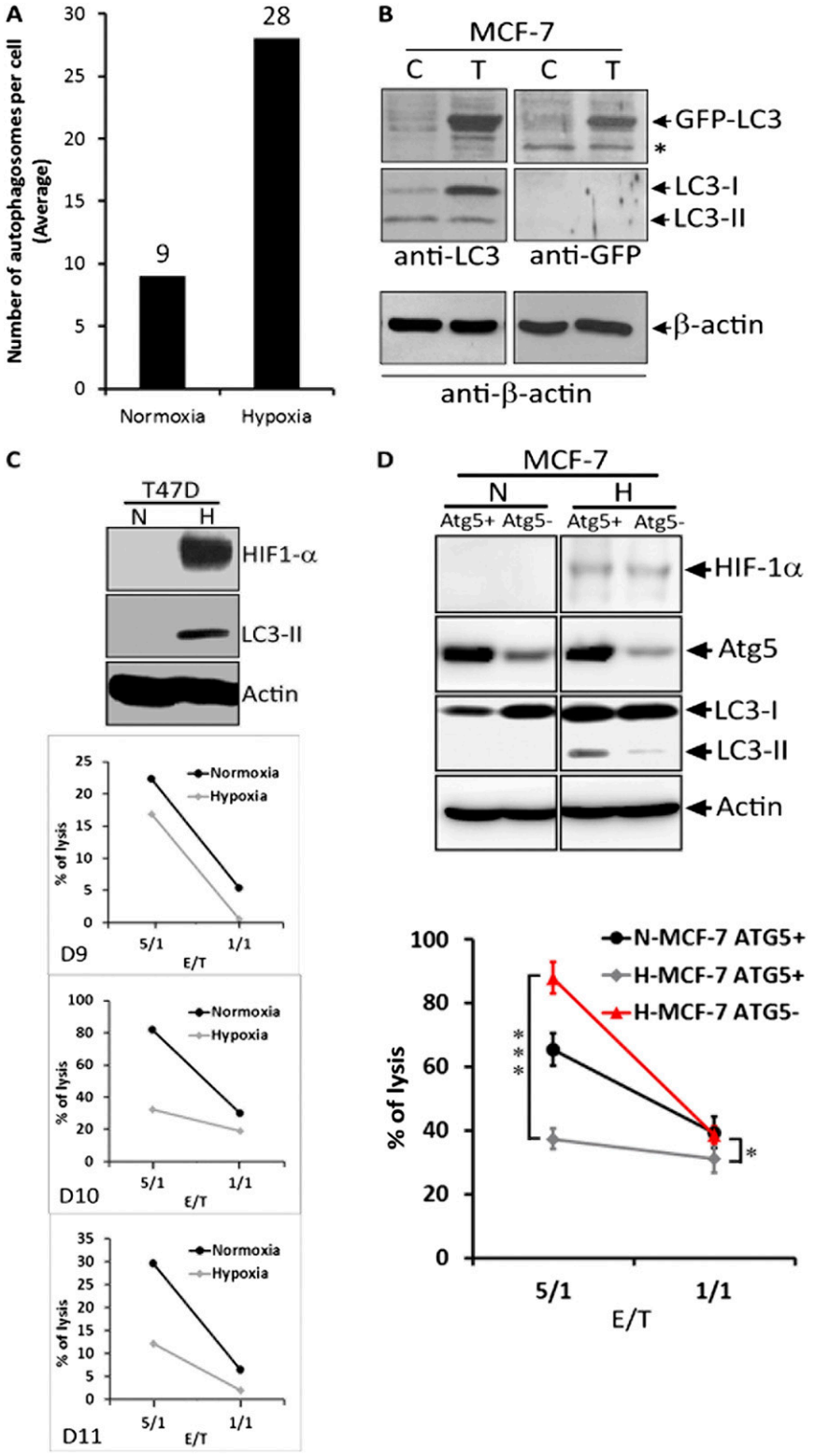


Fig. S1. (*A*) Quantification of the number of autophagosomes from representative images of normoxic and hypoxic MCF7 cells. The number of autophagosomes in 10 cells was counted and reported as an average number of autophagosomes per cell. (*B*) MCF-7 cells were stably transfected with GFP–LC3 cDNA and maintained in RPMI supplemented with 10% (vol/vol) FBS and geneticin (0.5 μg/mL). The expression of exogenous GFP–LC3 was assessed by immunoblot using anti-LC3 or anti-GFP antibodies, as indicated. An actin antibody was used for the loading control. C, control untransfected cells; T, cells transfected with GFP–LC3-encoding vector. The asterisk indicates an unspecific band. (*C*) Hypoxia-induced autophagy decreases the susceptibility of T47D breast cancer cells to NK-mediated lysis. (*C*, *Upper*) T47D cells were cultured under normoxia (N) or hypoxia (H) for 16 h. Expression of HIF-1α (hypoxia marker) and LC3 (autophagy marker) was assessed by immunoblot. (*Right*) NK cells were isolated from three different healthy donors (D9 to D11). Cytotoxicity assays were performed as described in Fig. 1*A*. (*C*, *Lower*) Cytotoxicity assays were performed as described in Fig. 1*A*. (*D*) Targeting ATG5 restores NK-mediated cytotoxicity against H

Legend continued on following page

MCF-7 cells. (*D*, *Upper*) Control (Atg5+) and ATG5-defective (Atg5−) MCF-7 cells were cultured under normoxic or hypoxic conditions. The expression of Atg5, HIF-1α, and LC3 was assessed by Western blot. (*D*, *Lower*) A cytotoxicity assay was performed at 5/1 and 1/1 E/T ratios using NK cells isolated from healthy donors as effector cells and control or ATG5–MCF-7 cells cultured under normoxic or hypoxic conditions as target cells. MCF-7 cell death was assessed using flow cytometry with TO-PRO-3. Statistically significant differences are indicated by asterisks (*$P < 0.05$; ***$P < 0.0005$).

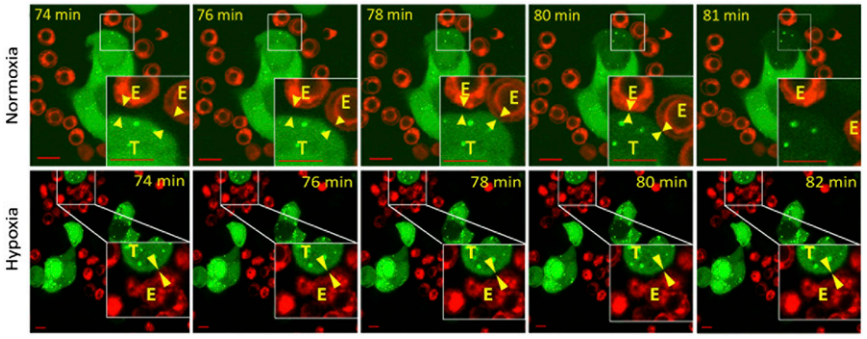


Fig. S2. GFP–LC3-expressing MCF-7 cells (green) were precultured onto glass coverslips under N or H conditions. The cells were incubated with PKH-26–stained NK cells (red) at a 5/1 E/T ratio. Interactions between NK and normoxic or hypoxic MCF-7 cells were recorded by time-lapse video microscopy (presented as Movies S1 and S2). At the indicated times, representative images were extracted from the movies. (Scale bar, 10 μm.) Images of enlarged regions (boxes) show the interaction between NK cells (E) with both N and H tumor cells (T). (Scale bar, 10 μm.)

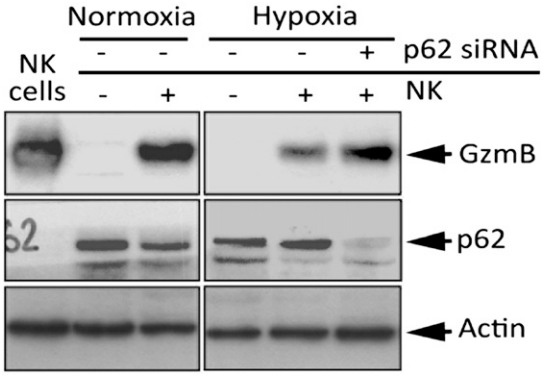


Fig. S3. Control (–) or p62/SQSTM1 siRNA transfected–MCF-7 (+) cells were cultured under normoxia or hypoxia for 16 h alone. Tumor cells were presented to NK cells (+) at 5/1 E/T ratio for 30 min. Following separation from NK cells, lysates of tumor cells were subjected to immunoblot analysis to evaluate the GzmB intracellular content. Untreated NK cells lysate (NK cells) was used as control for GzmB detection.

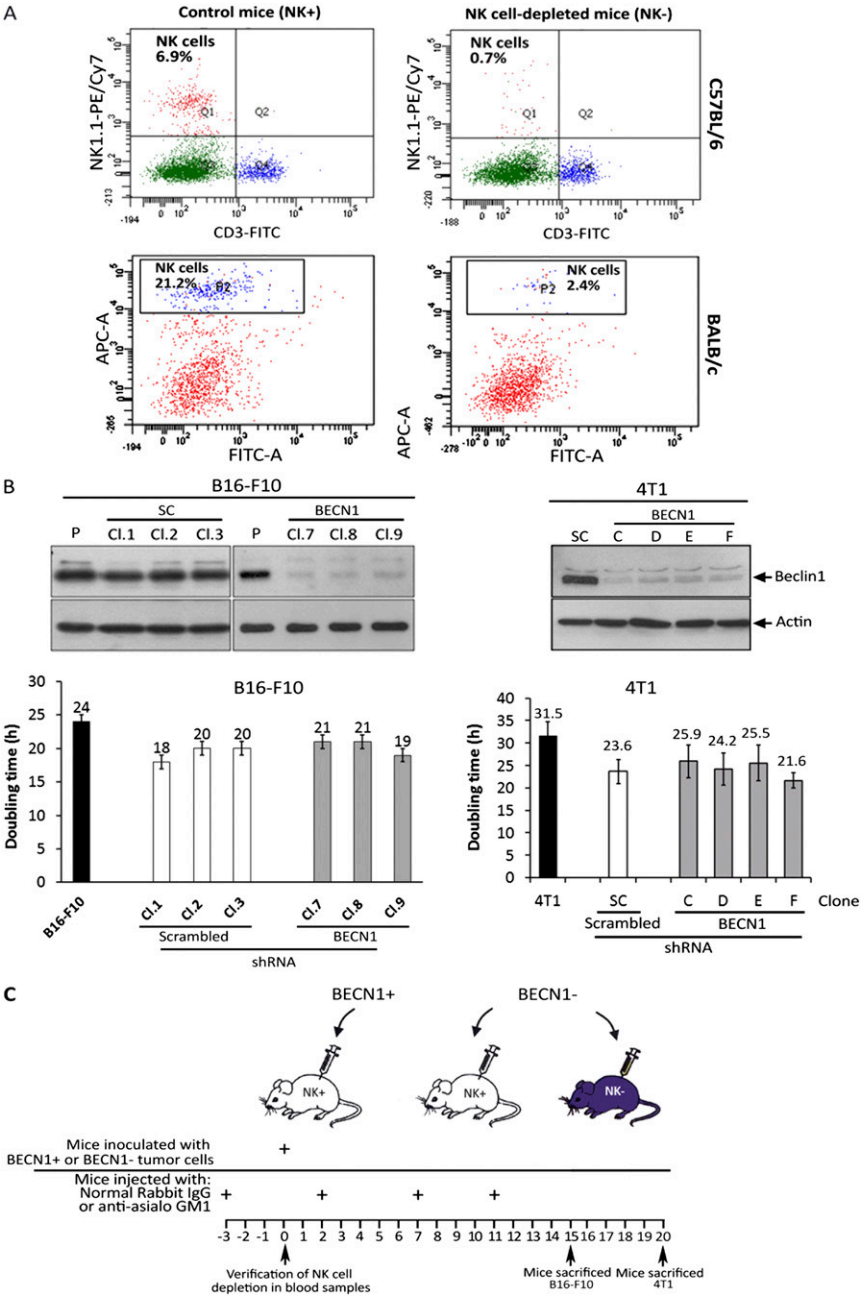


Fig. S4. (*A*) Validation of NK cell depletion in C57BL/6 and BALB/c mice. The effectiveness of NK cell depletion by antiasialo GM1 injection in C57BL/6 and BALB/c mice was determined using flow cytometric analysis. Representative dot-plots of NK1.1 and CD3 (C57BL/6) and CD49b (BALB/c) surface expression on PBMC from control (NK+) or NK-depleted (NK−) mice are shown. (*B*) Generation of autophagy-defective B16–F10 and 4T1 cells. Parental cells (P) were transduced with lentiviral particles containing scrambled (SC) or Beclin1 (BECN1) shRNA sequences. (*B*, *Upper*) SC and BECN1 B16–F10 and 4T1 clones were subjected to immunoblot analysis to evaluate the expression of Beclin1. (*B*, *Lower*) The doubling time of each clone was evaluated using the XTT assay ($n = 6$). (*C*) A schematic diagram of the experimental setup for in vivo experiments.

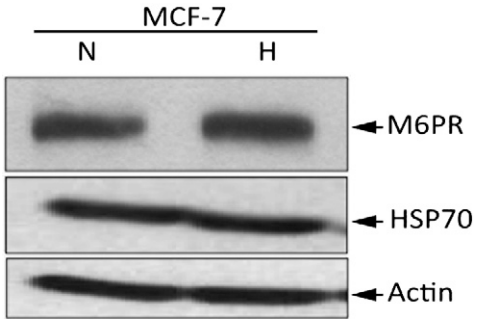


Fig. S5. Immunoblot analysis of the expression of M6PR and HSP70 in H and N MCF-7 cells. MCF-7 cells were cultured under normoxia (N) or hypoxia (H) for 16 h. The expression of M6PR and HSP70 was assessed by immunoblot.

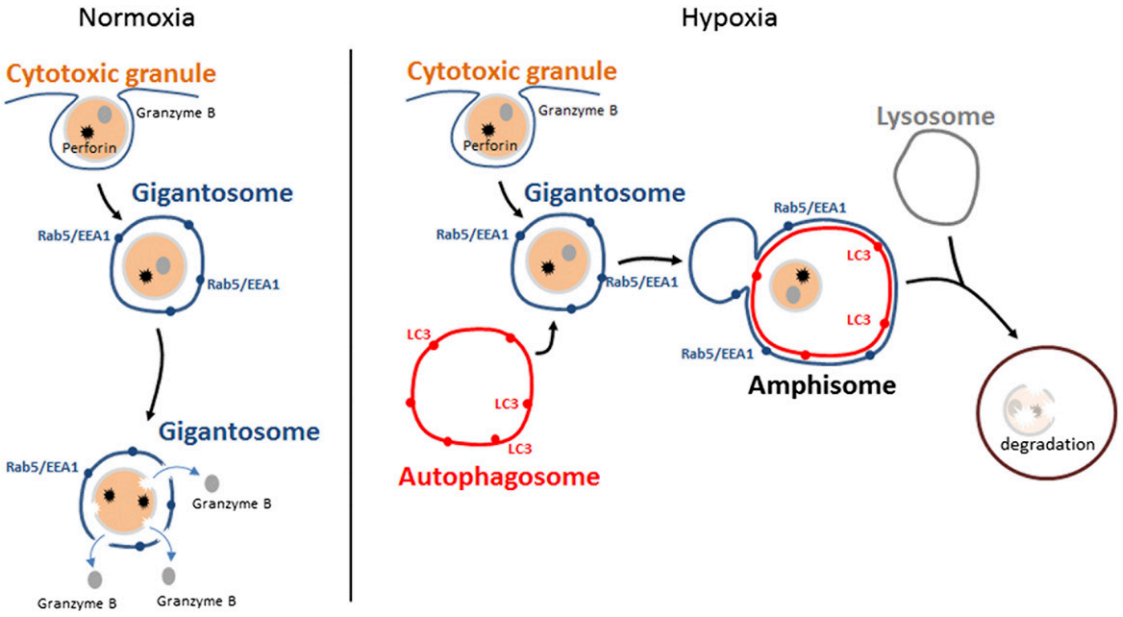
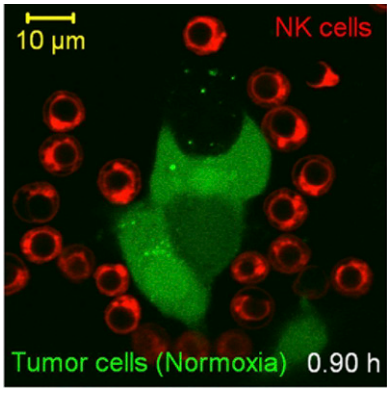
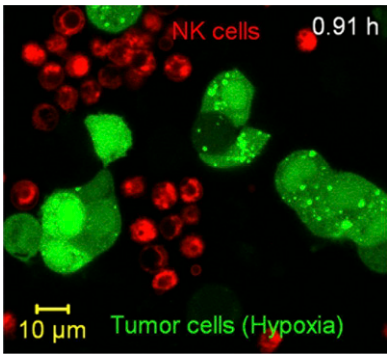


Fig. S6. Schematic representation of the mechanism underlying the degradation of GzmB by autophagy under hypoxia. Cytotoxic granules containing perforin and granzyme B are endocytosed into enlarged endosomes called "gigantosomes" in target cells. Under normoxia, perforin forms pores in the gigantosome membrane allowing granzyme B to be gradually released in the cytosol. In hypoxic cells the activation of autophagy leads to the formation of autophagosomes which will fuse with gigantosomes to form amphisomes. The fusion between amphisomes and lysosomes selectively degrades granzyme B in this compartment.



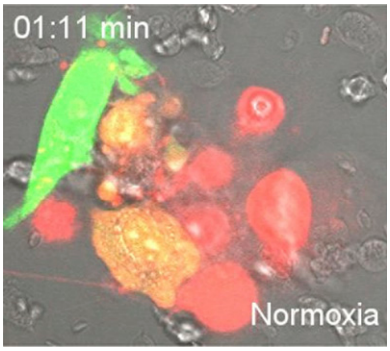
Movie S1. Time-lapse video microscopy of NK-mediated lysis of normoxic GFP–LC3–expressing MCF-7 cells. The movie was performed using N cells cultured on µ-Slide eight-well chambered coverslips (IBIDI) in the presence of PKH26-stained NK cells from a healthy donor (red) at a 5/1 E/T ratio. The movie was recorded for 1 h 22 min. Images were captured at intervals of one frame every 1–2 min with a Zeiss LSM-510 Meta laser-scanning confocal microscope (Carl Zeiss).

Movie S1



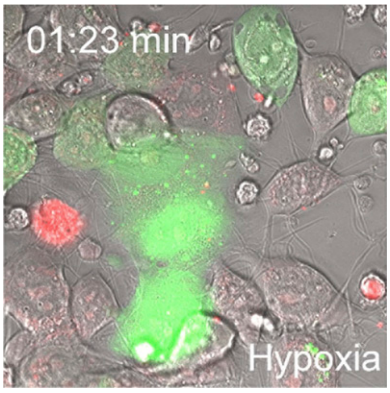
Movie S2. Time-lapse video microscopy of NK-mediated lysis of hypoxic GFP–LC3–expressing MCF-7 cells. The movie was performed using H cells cultured on μ-Slide eight-well chambered coverslips (IBIDI) in the presence of PKH26-stained NK cells from a healthy donor (red) at a 5/1 E/T ratio. The movie was recorded for 1 h 26 min. Images were captured at intervals of one frame every 1–2 min with a Zeiss LSM-510 Meta laser-scanning confocal microscope (Carl Zeiss).

Movie S2



Movie S3. Time-lapse video microscopy of NK-mediated lysis of normoxic GFP–LC3–expressing MCF-7 cells in the presence of cell death indicator. The movie was performed using normoxic cells cultured on μ-Slide eight-well chambered coverslips (IBIDI) in the presence of propidum iodide (PI) (1.5 μM) and NK cells from a healthy donor at a 5/1 E/T ratio. Tumor cell killing was recorded by time-lapse video microscopy using AxioVert 200M microscope (Carl Zeiss Micro-Imaging), and images were captured using ×40 oil objective lens at intervals of one frame per 20 s.

Movie S3



Movie S4. Time-lapse video microscopy of NK-mediated lysis of hypoxic GFP–LC3–expressing MCF-7 cells in the presence of cell death indicator. The movie was performed using hypoxic cells cultured on μ-Slide eight-well chambered coverslips (IBIDI) in the presence of propidum iodide (PI) (1.5 μM) and NK cells from a healthy donor at a 5/1 E/T ratio. Tumor cell killing was recorded by time-lapse video microscopy using AxioVert 200M microscope (Carl Zeiss Micro-Imaging), and images were captured using ×40 oil objective lens at intervals of one frame per 20 s.

Movie S4