Supplemental Information

Surface CD107a/LAMP-1 protects Natural Killer cells from degranulation-associated damage

André Cohnen$^{1,2}$, Samuel C. Chiang$^3$, Ana Stojanovic$^4$, Hendrik Schmidt$^5$, Maren Claus$^{1,2}$, Paul Saftig$^6$, Ottmar Janßen$^5$, Adelheid Cerwenka$^4$, Yenan T. Bryceson$^3$ and Carsten Watzl$^{1,2}$
Figure S1: Analysis of CD107a, CD107b, CD63 and perforin expression in T and NK cells.

(A) Purified human peripheral blood lymphocytes were permeabilized and stained for CD107a, CD107b, CD63 and perforin (Pfn). Mean fluorescent intensity (MFI) of the respective staining was analyzed in different subpopulations identified by the indicated surface markers. (B) CD3–CD56<sup>bright</sup> (left) and CD3–CD56<sup>dim</sup> (right) NK cells or (C) naïve CD3⁺CD8⁺CD27⁺ and effector CD3⁺CD8⁺CD27⁻ cytotoxic T cells were left untreated (white bars) or stimulated (black) with K562 targets (B) or P815 cells coated with anti-CD3 antibody (C) in the presence of anti-CD107a, anti-CD107b or anti-CD63 F(ab')<sub>2</sub> fragments. Surface expression was then analyzed by flow cytometry. Data represent means generated from data of 6 healthy donors ± s.e.m.
Figure S2: The HAGY motif controls lysosomal targeting of CD107a.

(A) Schematic representation of the CD107a protein. The position of the HAGY motif and the introduced stop codon are indicated. White arrows: N-linked glycosylation sites; black arrows: O-linked glycosylation sites; SP: signal peptide; TM: transmembrane domain. (B) Confocal image of HeLa cells expressing surface CD107a. (C) Alignment of the CD107a and CD107b hinge regions. The numbers indicate the amino acid positions. Identical amino acids are marked by black boxes, similar ones are shaded. Identical (red) and differing (black) O-glycosylation sites are marked by an asterisk.
Figure S3: localization of sCD107a in conjugates.

HeLa mock (A) or HeLa sCD107a (B) were plated in chamber slides at a density of 40,000 cells per well. IL-2 activated primary NK cells were added at an E/T ratio of 2/1 and incubated for 15’ to allow conjugate formation. Subsequently, cells were stained with FITC-conjugated anti-CD107a antibody (shown in green), fixed and counterstained with DAPI (blue).

(C) Mean fluorescence intensity of anti-CD107a staining was determined along a line drawn across the NK – HeLa contact area and a distal HeLa cell membrane area. MFI at contact area and distal area was determined as area under curve (AUC) of signal peaks corresponding to the contact site and distal HeLa cell membrane.
Figure S4: Conjugate formation of NK cells with surface CD107a expressing cells is unaffected.

(A) PKH red stained mock or CD107a transfected HeLa cells and PKH green stained IL-2 activated NK cells were mixed at an E/T ratio of 0.5. After co-incubation for the indicated time points, cells were vortexed and fixed with 2% PFA. Conjugates were determined as double positive events by FACS analysis. Shown is one representative out of 3 independent experiments. (B) Surface CD107b does not mediate target cell protection. IL-2 activated human NK cells were incubated with HeLa target cells, which stably expressed surface CD107b (filled triangles), CD107a (open triangles) or Clec12B as a control (mock, squares). Specific lysis was assessed by $^{51}$Cr release after 4 h of co-incubation at different effector to target (E/T) ratios. Values represent triplicates ± SD, n = 4.
Figure S5: Scheme of CD107a mutants used. Top: Boxed area represents the hinge region. Dotted line: Regions upstream and downstream of the hinge, the N-terminus is left. O-glycosylated amino acids are bold. Triple points indicate spacers longer than one amino acid. Bottom: Scheme outlining the domain order of the chimeric mutants. SP: Signal Peptide, TM: transmembrane helix.
**Figure S6:** Surface expression of (a) single, (b) double, (c) triple and (d) quadruple/chimeric CD107a mutants after cell sorting. Light grey: Isotype control, dark grey: staining of HeLa sCD107a WT, black line: staining of sCD107a mutant. For details on mutants see Fig. S4.
**Figure S7: Analysis of the role of CD107a associated O-glycans.**

IL-2 activated human NK cells were incubated with HeLa cells stably expressing the indicated sCD107a mutants. Specific lysis was assessed by $^{51}$Cr release after 4 h of co-incubation at different effector to target (E/T) ratios. % Inhibition was calculated as stated in Materials and Methods. Shown is the Mean ± s.e.m.. Each dot represents a single value out of triplicates from 5-13 experiments of an E/T of 10/1. For details on the mutants see Supplementary Fig. 4. Significances were analyzed using one-way ANOVA ( $P < 0.0001$), followed by Bonferroni’s Multiple comparison, ** = $P < 0.01$, *** = $P < 0.001$. $n = 20$ (mock), 18 (CD107a/b), 8 (T$_{189}$; HoB), 7 (SxTT; no O-Glycs), 5 (SxSxS; SxS$_{218}$; T$_{189}$SxSxS; SxSxS, SxS$_{218}$; SxTT-SxS$_{218}$; T$_{189}$SxSxS), 4 (T$_{189}$SxTT; HoA; SxTT, SxSxS; BBA; ABB; BAA), 3 (SxTT, SxS$_{216}$; T$_{189}$SxTxT+SxS$_{218}$; T$_{189}$+SxSxS+SxS$_{218}$; AAB).
Figure S8: Analysis of CD107a−/− NK cells.

(a) Splenic NK cells were purified from WT (white) or CD107a−/− (grey) C57BL/6J mice. The percentage of NKp46+CD3− splenocytes was determined. Statistical analysis was performed by two-tailed, unpaired Student’s t-test. (b) Surface expression of various surface markers on fresh (left) or IL-2 activated NK cells (right) purified from WT (white) or CD107a−/− (grey) C57BL/6J mice. Differences in expression levels are not significant, as determined by Student's t test. (c) IL-2 activated NK cells purified from WT C57BL/6J (white) or CD107a−/− mice (grey) were used as effector cells in ⁵¹Cr release assays against RMA/S targets at the indicated E/T ratios. Values represent mean from triplicates ± s.d., n = 4.
Supplemental Materials and Methods

Antibodies, Cells and Reagents - Antibodies used were: anti-CD107a PeCy5 (H4A3), FITC and PeCy7 conjugated anti-CD56 (clone NCAM16.2), anti-perforin FITC, Annexin-V PE (all from BD Pharmingen), control mouse IgG1 (clone MopC21), rabbit anti-actin (Sigma Aldrich), HRP conjugated goat anti-mouse and HRP goat anti-rabbit (Jackson Laboratories), anti-CD3 PerCPCy5.5 (clone 145-2C11) and anti-NK1.1 Alexa 647 (clone PK136, BioLegend). For staining of human PBMC, anti-CD3-PeCy5.5 (clone S4.1, Invitrogen), anti-CD8 Brilliant Violet 570 (clone RPA-T8, Biolegend), anti-CD27 Quantum Dot 655 (clone CLB-27/1, Invitrogen), anti-CD56-FITC and PE-Cy7 (clone NCAM16.2, BD Bioscience), anti-CD63-FITC (clone H5C6, BD Bioscience), anti-CD107a-FITC (clone H4A3, BD Bioscience), anti-CD107b-FITC (clone H4B4, BD Bioscience), and anti-perforin FITC (clone δG9, BD Bioscience) were used.

Peripheral blood was obtained with informed consent from healthy blood donors at the Karolinska University Hospital. The study was approved by the regional ethical board. Data was acquired on a LSR Fortessa flow cytometer (BD Bioscience) and analyzed with Flowjo software (v9, Treestar).

Arthrobacter ureafaciens α2-3,6,8,9-neuraminidase and BADG (Benzyl-2-acetoamido-2-deoxy-α-D-galactopyranoside) were from Merck-Calbiochem (Darmstadt, Germany).

Reverse Transcription PCR and cDNA Constructs - CD107a and CD107b cDNA was amplified from primary human NK cells using the primer pairs GAG GAG GTC GAC ATG GCG GCC CCC GGC AGC G; CTC CTC GGA TCC CTA ACT CCT CTT CCT GCC
GAC GAG GTA GGC G and GAG GAG GTC GAC ATG GTG TGC TTC CGC CTC TTC CC;  
CTC CTC GGA TCC CAT GGT GCT TGA GAC CAA TAA AAT AAG CCA GC,  
respectively. CD107a was cloned into the retroviral expression vector  
pMOWplus, CD107b was linked to a GFP tag by in-frame subcloning into  
pMOWplus eGFP. HeLa and 721.221 cells were retrovirally transduced and  
selected in puromycin and FACS sorted for stable expression of CD107a and  
CD107b::GFP respectively. Expression was verified by flow cytometry.  
Mutagenesis of single O-glycan domains was performed using appropriate  
primers. Double and triple mutants were produced by site-directed mutagenesis  
of existing mutants. Domain swapping was performed using overlap-extension  
on HoA and HoB constructs.

*FACS based conjugate assay* - FACS based conjugate formation was carried out as  
described earlier  
1. Briefly, NK cells and target cells were labeled with PKH26 and  
PKH67, respectively (both Sigma) and resuspended in cold IMDM medium.  
5 x 10^4 effector cells were mixed with target cells in 100 μl at an E/T of 0.5,  
centrifuged for 2 minutes at 20 x g and co-incubated at 37°C for the indicated  
time-intervals. Subsequently, cells were extensively vortexed, fixed with 4%  
paraformaldehyde in PBS and measured on a FACScan. Number of double  
positive events compared to single NK cell events and multiplying by 100  
determined percent NK cells in conjugate.

*Purification of cytotoxic granules* - Cytotoxic granules were enriched from  
leukemic NK cell lines (YTS and NKL) following the recently described procedure  
2,3. The cells (1.14x10^9 YTS, 1.54x10^9 NKL) were washed with ice-cold PBS,  
resuspended in 6 ml extraction buffer including protease inhibitors (Sigma) and
carefully disrupted in a dounce glass homogenizer. Homogenates were centrifuged at 1,000 x g for 10 min to separate nuclei and remaining intact cells. Organelles were enriched from the supernatants by centrifugation at 20,000 x g for 20 min. The resulting pellet was resuspended in a small volume of extraction buffer, adjusted to 19% (v/v) Optiprep (Sigma), placed in the middle of a discontinuous density gradient with 27%, 22.5%, 19%, 16%, 12%, 8% OptiPrep and centrifuged at 150,000 x g for 5 h in a SW60Ti swing-out rotor (Beckman Coulter, Krefeld, Germany). Individual fractions containing effector granules were collected from the top of the tube and kept frozen until further use.

*siRNA transfection* – 3 x 10^6 primary IL-2 activated NK cells were transfected with 10 pM siRNA (Lamp1 ON-TARGETplus SMART pool and ON-TARGETplus non-targeting siRNA, Dharmacon) using the Amaxa Macrophage Nucleofector kit, program X-001 according to the manufacturer’s manual. Subsequently, cells were cultured in medium containing 100 U/ml IL-2 and 5 pg/ml IL-15. Knockdown efficiencies were tested by immunoblotting. siCD107a or siCTRL NK cells were mixed with or without K562 target cells at an E/T of 0.5 and co-incubated for 4 h in the presence of PeCy5 conjugated anti-CD107a antibody. Afterwards, cells were stained with PeCy7 conjugated anti-CD56 for 20 min on ice. Subsequently, cells were washed, resuspended in Annexin-V binding buffer and stained with PE conjugated Annexin-V according to the manufacturer’s protocol. Cells were analyzed in a BD LSR2 and gated on CD56+ events. Fold increase in dead cells per gate was calculated as $\frac{MFI_{siCD107a\ (Annexin-V)}}{MFI_{siCTRL\ (Annexin-V)}}$.

*Deglycosylation of 721.221 cells* - Desialylation of cells was done using 7.5 µl neuraminidase for 1 x 10^6 cells/100 µl PBS rotating 2 h at 37 °C/5 % CO₂. To
inhibit elongation of O-glycosyl chains, cells were incubated with 3 mM BADG for 48 h at 37 °C/5 % CO₂. Cells were washed once with supplemented IMDM medium and used for cytotoxicity assays as described.

References