Supplementary Information

NK cell detachment from target cells is regulated by successful cytotoxicity and influences cytokine production

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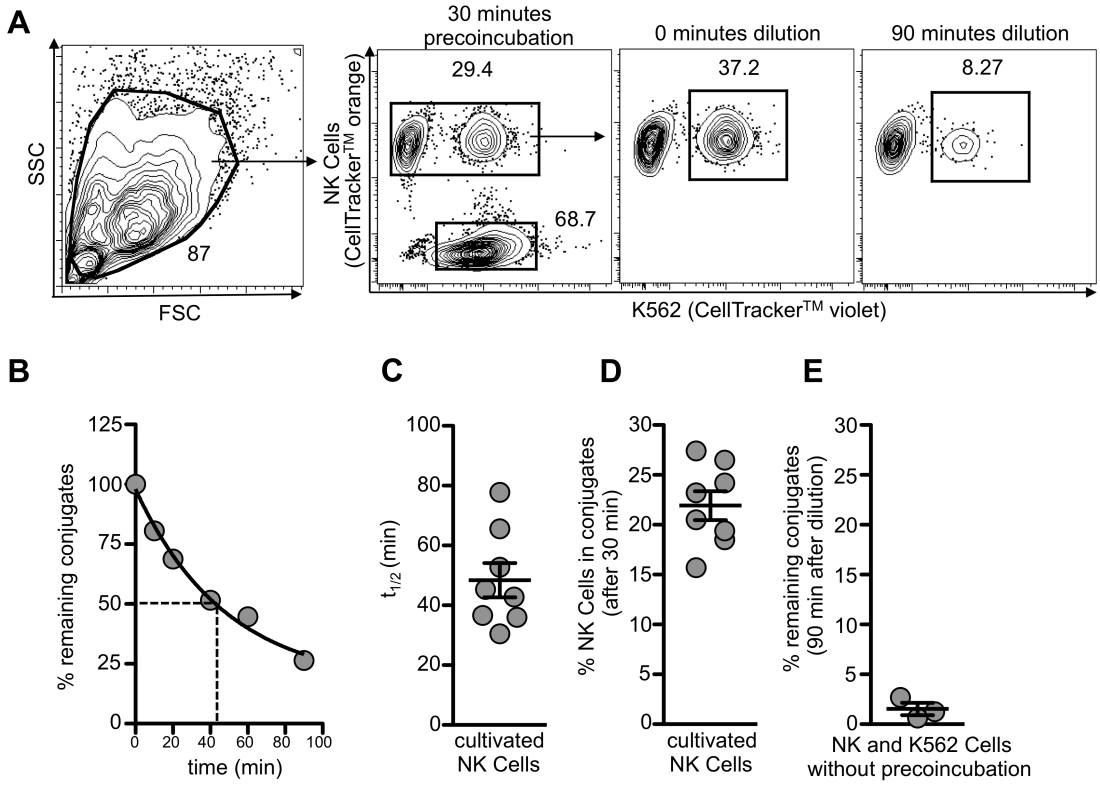

Figure S1. Flow cytometry-based detachment assay. (**A**) Gating strategy of the detachment assay: NK cells (stained with CellTracker orange) were mixed with K562 cells (stained with CellTracker violet), analyzed by flow cytometry and gated as shown. Plots are shown for 0 and 90 minutes after dilution and agitation. (**B**) The percentage of NK cells in conjugates was determined for all time-points (0, 10, 20, 40, 60 and 90 minutes), normalized to time point 0 minutes after pre-coincubation and plotted against the time. (**C**) Using the formula for one-phase exponential decay the half-life for the NK cell detachment was calculated and quantified for cultivated NK cells from 8 different healthy donors. (**D**) The number of conjugates of NK cells from 8 different healthy donors after 30 minutes pre-coincubation with K562 targets. (**E**) To demonstrate that the dilution and agitation by rotation prevents the formation of new conjugates, NK and K562 cells were mixed without pre-coincubation. Shown is the number of conjugates after 90 minutes of agitation in diluted medium.

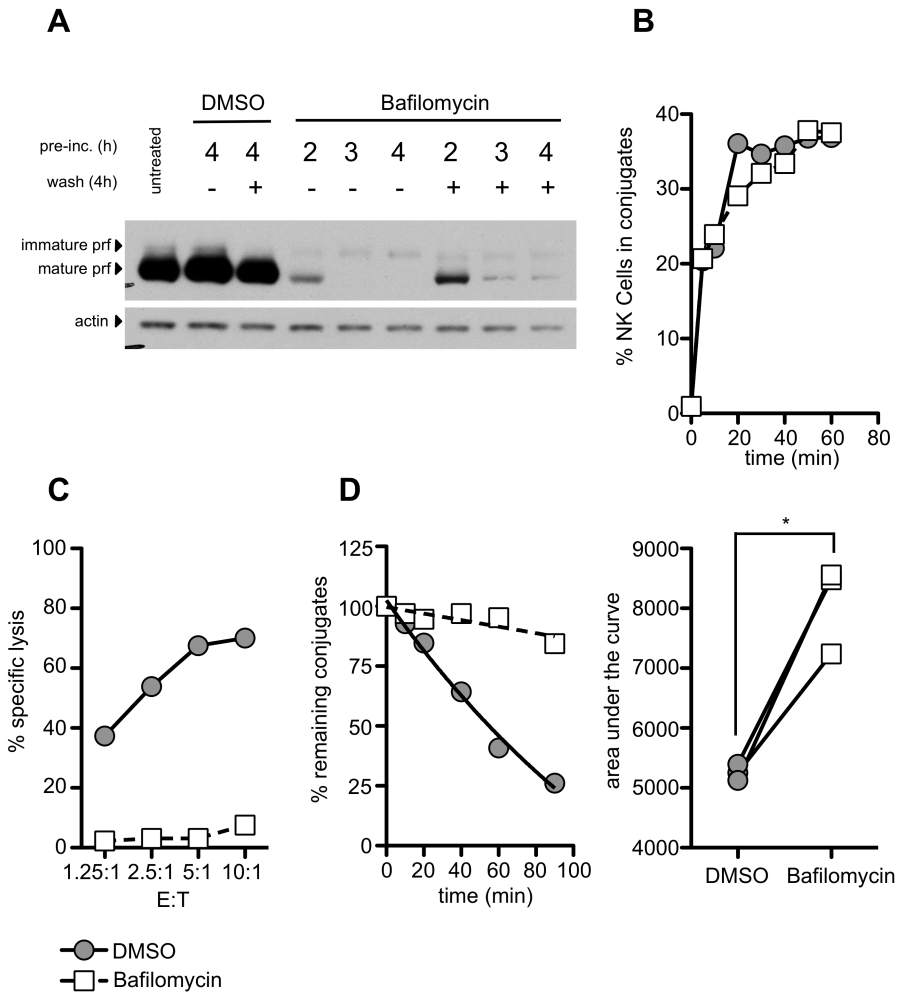

Figure S2. Bafilomycin A1 reversibly depletes mature perforin, reduces target cell lysis and decelerates NK cell detachment. (**A**) NK cells were treated with bafilomycin or solvent control for 3 h. Subsequently, the cells were washed and incubated for up to 4 h with (+) or without (-) the inhibitor present. Cell lysates were analyzed for perforin (prf1) expression by western blot and actin was used as a loading control. (**B,C,D**) NK cells were pre-treated with bafilomycin or DMSO for 3 h, washed and used in the assays without the inhibitor present. (**B**) Formation of conjugates was tested in a conjugate assay, **(C)** cytotoxicity was tested in a standard 4 h chromium release assay. **(D)** Detachment of NK cells was analyzed in a detachment assay. Left panel shows one representative experiment, right panel shows the quantification of three independent experiments with different healthy NK cell donors (*p<0.05). We used the area under the curve (AUC) to compare the samples as no detachment half-life for the Bafilomycin A1-treated samples could be determined.

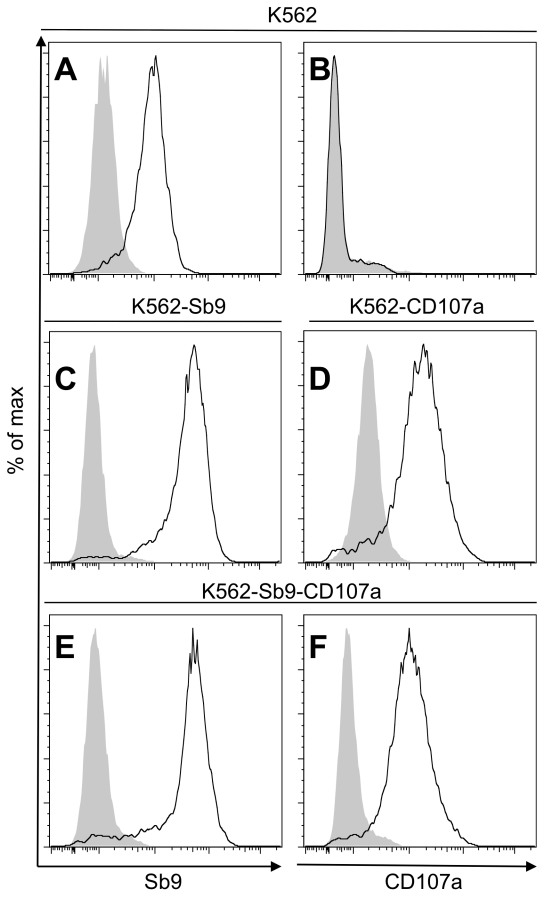

Figure S3. **Expression of SerpinB9 and surface-CD107a on the indicated K562 transfectants.** K562 cells were retrovirally transfected with a SerpinB9- and/or surface-CD107a-Vector. Cells were stained with α-SerpinB9 (left panels) or α-CD107a antibody (right panels). Grey histograms show staining of the respective cells with IgG antibody as a control.

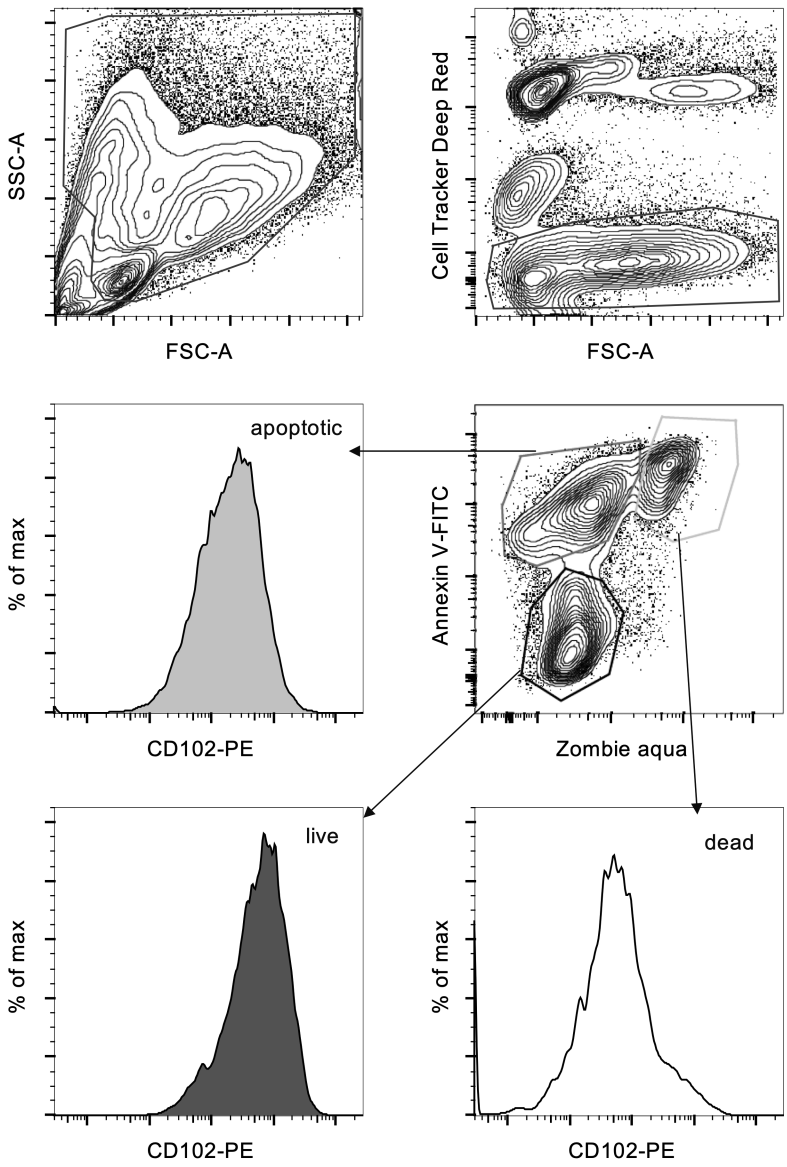

Figure S4. Gating strategy to analyze the expression of activating ligands and adhesion proteins on living, apoptotic and dead K562 cells. Cultivated NK cells were stained with Cell tracker deep red and co-incubated for 2 h with K562 cells. Afterwards, all cells were stained with antibodies against the indicated proteins (here CD102), with life/dead marker Zombie Aqua™ and Annexin V. Within the free K562 cells, gates were set for Zombie and Annexin V negative (living), Annexin V single positive (apoptotic) and Annexin V and Zombie positive (dead) K562 cells.

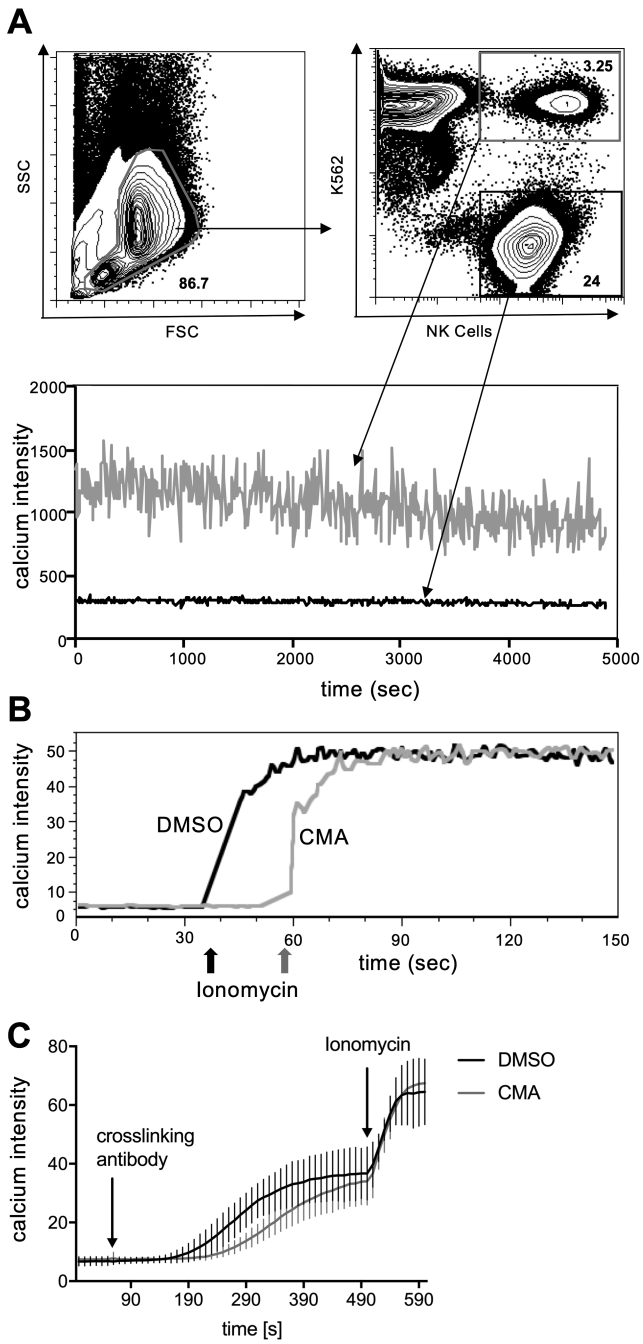

Figure S5. The calcium signal of NK cells in conjugates is increased compared to free NK cells. (A) NK cells were stained with calcium binding dye fluo-8 and K562 cells with cell tracker orange to distinguish between free NK cells, free K562, and conjugates. NK cells were co-incubated with K562 target cells for 30 minutes (E:T = 1:2). The calcium signal of free NK cells (lower gate, black line) and NK cells in conjugates (upper gate, grey line) was analyzed over 80 minutes. **(B)** NK cells were treated with CMA or DMSO as solvent control and loaded with fluo-8. Calcium flux was analyzed by flow cytometry and 1µM ionomycin was added at the indicated time point. **(C)** NK cells were treated with CMA or DMSO as solvent control and loaded with fluo-8. Cells were stained with antibodies against NKG2D and 2B4 and calcium flux was analyzed by flow cytometry. At the indicated time points secondary antibodies were added to crosslink NKG2D and 2B4 and ionomycin was added as positive control.

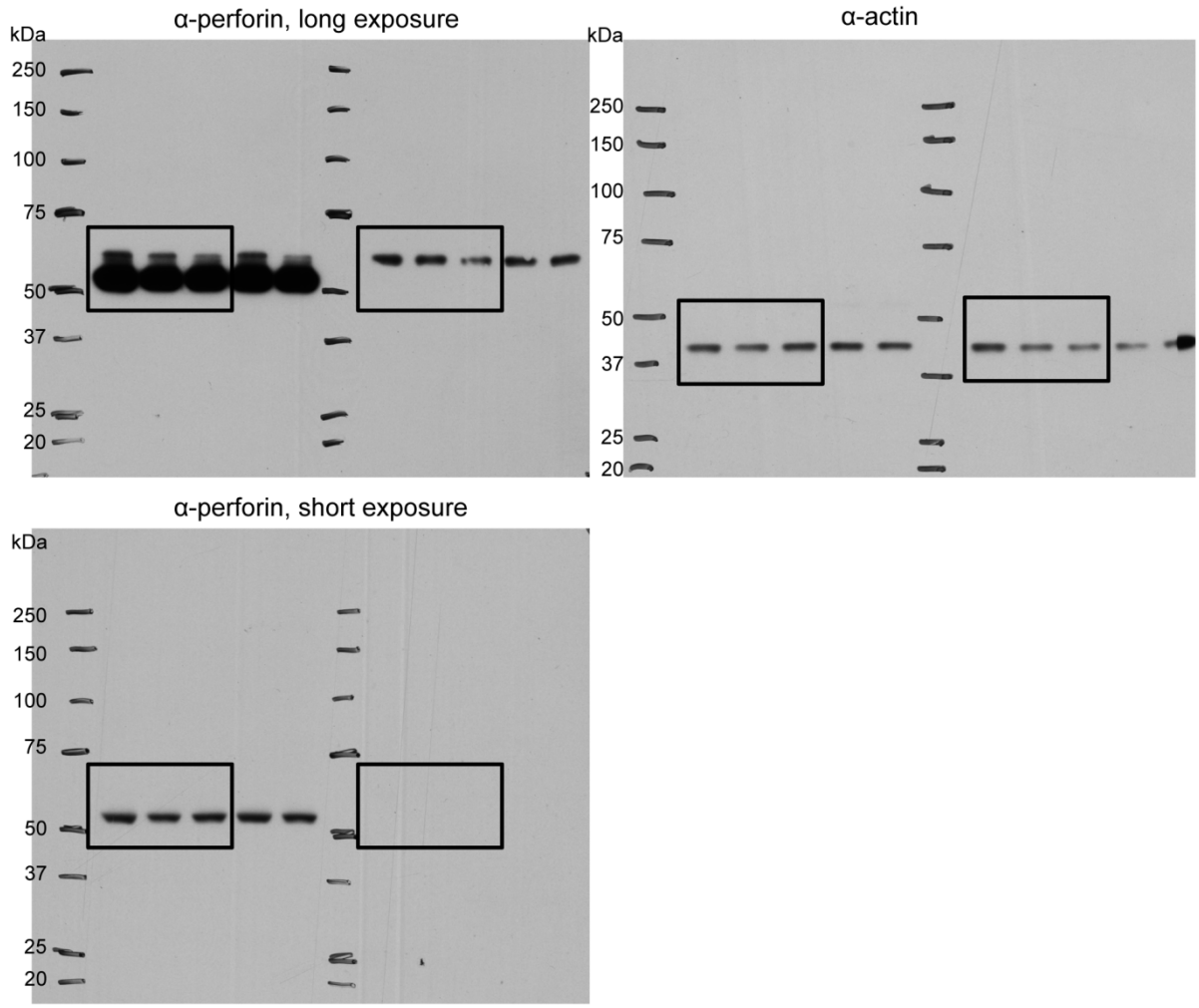

Figure S6. Original Western blots of concanamycin A or DMSO treated NK cells. Boxed areas are shown in figure 2.