Natural killer cells induce distinct modes of cancer cell death: Discrimination, quantification and modulation of apoptosis, necrosis and mixed forms


Christian S. Backes¹, Kim S. Friedmann¹, Sebastian Mang¹, Arne Knörck¹, Markus Hoth¹*, Carsten Kummerow¹*

From the ¹Department of Biophysics, Center for Integrative Physiology and Molecular Medicine, School of Medicine, Saarland University, 66421 Homburg, Germany.

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* To whom correspondence should be addressed:
Carsten Kummerow, Biophysics, Center for Integrative Physiology and Molecular Medicine, Saarland University, 66421 Homburg, Germany; carsten.kummerow@uks.eu; Tel. +49 6841 1616318; Fax. +49 6841 1616302.
Markus Hoth, Biophysics, Center for Integrative Physiology and Molecular Medicine, Saarland University, 66421 Homburg, Germany; markus.hoth@uks.eu; Tel. +49 6841 1616303; Fax. +49 6841 1616302.

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ABSTRACT

Immune therapy of cancer is among the promising recent advances in medicine. Whether the immune system can keep cancer in check depends, among other factors, on the efficiency of immune cells to recognize and eliminate cancer cells. We describe a time-resolved single cell assay which reports quality, quantity and kinetics of target cell death induced by single primary human natural killer (NK) cells. The assay reveals that single NK cells induce cancer cell death by apoptosis and necrosis but also by mixed forms. Inhibition of either one of the two major cytotoxic pathways, perforin/granzyme release or FasL/FasR interaction, unmasked the parallel activity of the other one. Ca²⁺ influx through Orai channels is important for tuning killer cell function. We found that the apoptosis to necrosis ratio of cancer cell death by NK cells is controlled by the magnitude of Ca²⁺ entry and furthermore by the relative concentrations of perforin and granzyme B. The possibility to change the apoptosis to necrosis ratio employed by NK cells offers an intriguing possibility to modulate the immunogenicity of the tumor microenvironment.

State-of-the-art immunotherapy against cancer includes CAR T cell therapy (1,2), checkpoint inhibitor therapy (3,4) or other antibody-based therapies (5,6) such as rituximab against CD20⁺ lymphoma (7,8). Cancer therapies commonly converge on the cytotoxic arm of the immune system (3,9,10). Both, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are involved in the immune surveillance of cancer. While CTL recognize specific peptides presented by MHC-I using the T cell receptor, NK cells analyze both activating and inhibitory ligands on the target cell with their germline-encoded receptor repertoire and integrate these signals. Upon activation, killer cells form a tight contact with the target called the immune synapse (IS) and polarize lytic granules (LG) as well as other
organelles and molecules towards the contact site (11,12). LG contain the pore-forming protein perforin which, upon release, generates holes in the target cell membrane and allows serine proteases called granzymes to enter the cytosol of the target cell. If this local membrane disruption cannot be neutralized by the target cell, cell death by rapid swelling and lysis follows (necrosis). If membrane integrity can be restored, the injected granzymes induce cell death by caspase activation and subsequent caspase-dependent apoptosis (13). In addition to the granule-based killing pathway, another well-established killing mechanism involves the interaction of Fas-ligand (FasL) on cytotoxic cells with Fas-receptor (FasR)-positive target cells inducing target cell apoptosis (14). While CAR T cell therapy is well established in the clinic for several years, much work is currently focused on exploring the anti-tumor potential of primary human natural killer (NK) cells and also the cell line NK-92 in the clinic (9,15).

Mal-functioning of NK and CTL’s perforin is, in its severest case, associated with life-threatening hyperinflammation as part of Hemophagocytic Lymphohistiocytosis (HLH) (16). While the complete absence of perforin function is a rare disease presenting with severe symptoms during infancy (Familial hemophagocytic lymphohistiocytosis 2, FHL2), hypomorphic mutations with reduced perforin concentration or function are more frequent in the population (16,17). While not immediately life threatening, a reduced perforin function is linked with increased susceptibility to late-onset HLH (16,18) and development of cancer (17-19). These findings highlight the importance of analyzing NK cell and CTL cytotoxicity in a detailed and quantitative manner.

Results

Casper-GR as a single cell apoptosis marker

Jurkat E6-1 and K562 are well-characterized targets for NK cytotoxicity. The FRET-based apoptosis reporter Casper3-GR (20) was stably transfected into both cell lines to create stable, monoclonal target cell lines named Jurkat pCasper and K562 pCasper to analyze NK-cell mediated cytotoxicity on a single cell level. Casper-GR consists of a green fluorescent protein (GFP) and a red fluorescent protein (RFP) fused by a linker containing the caspase recognition sequence DEVD. Since the DEVD sequence is not caspase-3 specific but cleaved by caspase-3 and -7 as well as other caspases (21,22), we prefer to refer to the construct as Casper and not Casper3 to acknowledge this fact. Due to the close proximity of GFP and RFP in Casper-GR and their matched emission and excitation spectra, a considerable part of the GFP energy will be transferred to RFP in a radiationless manner by Försters Resonance Energy Transfer (FRET), leading to red fluorescence upon excitation with blue light. Upon cleavage of the linker by caspase activity, this red fluorescence will decrease as a result of the increasing distance of the fluorophores, while an increase in green fluorescence will be detected. These two target cell lines were chosen because they greatly differ in their expression of the Fas receptor (FasR) CD95. Jurkat E6-1 cells express FasR and K562 do not express FasR (23,24).

To test the viability of Jurkat pCasper and K562 pCasper cells, both cell lines were imaged for 6 h at 37 °C, 5 % CO₂ acquiring GFP-, RFP- and FRET emission. Counting dead cells after 6 h revealed, that under these conditions 4.1% of Jurkat pCasper and 1.8% of K562 pCasper cells (n = 296 or n = 284, respectively) showed signs of apoptosis (Fig. 1a, b), comparable to control cells. Thus, monoclonal viable Jurkat pCasper and K562 pCasper cells positive for both, GFP and RFP expression (Fig. S1) were successfully generated.

Using flow cytometry analysis and qRT-PCR, we confirmed the findings by others (23,24) that Jurkat E6-1 but not K562 cells express FasR (Fig. S2a, b, Table S1). Importantly for our study, this expression profile was not changed by stable pCasper-GR transfection (Fig. 2c, d).

To verify pCasper-GR functionality, Jurkat pCasper (Fig. 1c) or K562 pCasper (Fig. 1d) cells were incubated for 6 h with the apoptosis inducer staurosporine (2.5 µM) or the CD95 activating antibody Apo 1-1 (5 µg/ml). In both cell lines, staurosporine caused cell death (Fig. 1c, d upper panels) which was verified by cell blebbing and cell shrinking. Fluorescence intensity decreased in the FRET channel (red) and correspondingly increased in the GFP channel (green), indicating caspase dependent cleavage of the recognition sequence DEVD. The functionality of the CD95 antibody Apo 1-1, however, was restricted to Jurkat pCasper cells (Fig. 1c, lower panel). K562 pCasper cells showed no sign of cell death.
following Apo 1-1 incubation and cells remained orange (Fig. 1d, lower panel). These results confirm functionality of Jurkat pCasper and K562 pCasper cell lines to specifically measure caspase dependent apoptosis and also provide further functional evidence for the presence of FasR in Jurkat pCasper but not in K562 pCasper cells.

To define time points of apoptosis induction, we analyzed the FRET donor ratio according to Youvan et al. (25), which has the great advantage that it is intensity independent. Fig. 1e shows the time course of an experiment similar to the one shown in Fig. 1d. Application of 2.5 μM staurosporine induces the change from orange to green fluorescence in K562 pCasper cells at different time points (4 cells are marked R1 to R4). The FRET signal and the FRET donor ratio after Youvan et al. were calculated (both color coded). Fig. 1f shows that in the donor ratio time series, the signal of cells R1 – R4 disappear at different time points but remain constant before this time point. The change in slope can be easily quantified and is a very reliable measure for apoptosis induction. This method was used to quantify data as shown in Fig. 1a-d. The results are shown in Fig. 1g and represent the time course of apoptosis induction in Jurkat pCasper and K562 pCasper cells by staurosporine or Apo 1-1. In Jurkat pCasper cells, both staurosporine and Apo 1-1 induce apoptosis without much delay. In K562 pCasper cells, staurosporine induces apoptosis after a delay of 2-3 hours. Apo 1-1 has no effect which is expected considering the absence of the FasR CD95.

To test the specificity of the Casper-GR construct, we mutated its DEVD binding site to DEVA which cannot be cleaved by caspase. Jurkat E6-1 cells were transiently transfected and exposed to the same Apo 1-1 or staurosporine concentrations as in Fig. 1c. Two representative cells are shown in Fig. 1h. Despite clear morphological signs of apoptosis induction (blebbing and loss of organelle movement), there was no change in the overlay of FRET fluorescence and GFP signal of Caspase3-GR (upper panel) or in the donor ratio (lower panel) as also evident from the average of 10 cells for each condition (Fig. 1i). We did not observe a single transfected cell with any hint of fluorescence changes. In conclusion, Jurkat pCasper and K562 pCasper cells are reliable tools to report the exact time point of caspase-dependent apoptosis induction. The change of the caspase sensitive recognition sequence DEVD to the insensitive DEVA sequence rendered the FRET sensor inactive during apoptosis.

Casper-GR reports apoptosis and necrosis in target cells following contact with NK cells

Natural killer (NK) cells can induce apoptotic or necrotic cell death in their respective targets and potentially also induce “mixed” forms of target-cell death. A main difference between apoptotic and necrotic cell death is the integrity of the cell membrane, which is at least initially kept intact during apoptosis but immediately disrupted during necrosis (26).

Since Jurkat E6-1 and K562 cells differ in the expression of the FasR (23,24), they can be used to analyze NK cell mediated perforin/granzyme cytotoxicity either in combination with (Jurkat pCasper) or without (K562 pCasper) concomitant FasL/FasR induced apoptosis.

To quantify target cell death by primary human NK cells, Jurkat pCasper target cells were additionally loaded with fura-2, a small molecule calcium indicator with excitation spectra well separated from GFP and RFP. Fura-2 was excited close to its isosbestic point, where fluorescence does not depend on the calcium concentration. It was thus used here as a relatively small cytosolic dye to report target cell lysis by necrosis, since it will rapidly diffuse out of the cell once significant membrane damage has been inflicted. Fig. 2a shows three different fluorescence signals of the same fura-2 loaded Jurkat pCasper target cells: overlay of bright field signal and fura-2 fluorescence (upper panel); overlay of bright field signal, FRET fluorescence of Casper-GR and GFP signal of Casper-GR (middle panel); donor ratio (FRET/GFP, bottom panel). In this example, one NK cell approaches two target cells (marked by white arrows). The fura-2 signal sharply decreases at 0 min in target 1 once contacted by the NK cell (Fig. 2a), averaged in Fig. 2b for 5 targets. In parallel, the Casper-GR fluorescence signal starts to decline, albeit with slower kinetics due the large molecular weight of the sensor protein. These changes imply that target cell integrity is lost, indicative of necrosis. The donor ratio of target 1 (Fig. 2a, lower panel) is maintained over time with
no abrupt decrease, indicating that the target is not apoptotic.

The fura-2 signal of target 2 does not decrease over time, it even slightly increases (because the target slightly shrinks), but 5 min after contact with the same NK cell, the FRET signal of target 2 decreases which is paralleled by an increase of the GFP signal, indicative of a caspase dependent cleavage of the Casper-GR DEVD site, which diminishes the FRET efficiency and increases the GFP signal as averaged in 5 targets (Fig. 2c). Combining the drop of the FRET signal and the increase of the GFP signal, the donor ratio sharply drops (Fig. 2a, lower panel). Target 2 thus goes into apoptosis.

Since the rapid, parallel loss of both GFP and FRET fluorescence proved sufficient to determine killing by necrosis and because of potential harm to cell viability caused by UV light excitation, we proceeded to perform all subsequent imaging without Fura-2.

To further test membrane disruption as observed by dye loss during NK-cell induced killing of target 1, two dyes were combined as measured by Lopez et al. (27). Calcein was loaded in Jurkat E6-1 and propidium iodide was kept in the supernatant. Imaging revealed that after encountering a target cell, NK cells can induce the loss of the target cell’s cytosolic dye (calcein) and the parallel intrusion of the supernatant dye (propidium iodide, Fig. 2d and quantified in 2e). To verify that disruption of the target cell’s integrity is caused by the NK cell at the contact site, the fluorescence intensity of the intruding dye propidium iodide was analyzed. Using a line plot, which starts at the immune synapse (IS) and dissects the target cell in half (Fig. 2f), propidium iodide fluorescence intensity was plotted over time. Results confirm that propidium iodide enters the target cell at the IS and then gradually distributes to the distant part of the target cell (Fig. 2g) as described previously by Lopez et al. (27). This shows that NK cells are able to induce target necrosis by a disruption in the target cell’s membrane at the contact site, the IS.

In conclusion, Casper-GR can reliably be used as an apoptosis and necrosis sensor which reports single target cell death induced by primary human NK cells. Interestingly the same NK cell is able to kill two different targets within minutes by two different killing modes during serial killing, (see also Video 1).

**Distinguishing the target cell death spectrum by Casper-GR fluorescence pattern following NK cell contact**

Analyzing cell morphology and Casper-GR fluorescence in parallel offers the possibility to categorize the target cell death spectrum following contact with primary human NK cells. We have analyzed, classified and quantified different modes of cell death in Jurkat pCasper cells killed by human NK cells. Fig. 3a depicts a target cell which shows typical apoptosis signs after NK cell contact, it shrinks and blebs. This is paralleled by a prominent increase of GFP fluorescence and a decrease in the FRET signal. Quantification is shown in Fig. 3b. In particular the GFP signal increase and the concomitant donor ratio decrease offer an exact temporal quantification of apoptosis initiation as shown before in Fig. 2.

In some of the initially apoptotic target cells, a sudden drop of both the higher GFP signal (after apoptosis) and FRET fluorescence was observed (compare GFP fluorescence between 0 and 10 min in Fig. 3c). This was paralleled by a volume increase of the target cell, indicating that the cell was lysed. We refer to this type of cell death as secondary necrosis after the target cell was initially killed by apoptosis. Initiation of secondary necrosis can be exactly quantified by the abrupt decline in both GFP and FRET signals as indicated at time 0 in Fig. 3d whereas the donor ratio remains unchanged.

In target cells which show an increase of volume after NK cell contact without a hint for apoptosis (Fig. 3e), a parallel and quantitatively identical decrease of GFP and FRET signals was observed (Fig. 3f), indicating that target cell lysis was initiated. The donor ratio remains constant with no signs of apoptosis. We refer to this type of cell death as primary necrosis because cells eventually lose their integrity and burst open (without any hint for apoptosis).

In some target cells we observed a mix of morphological signs indicative of apoptosis and necrosis in the target cells (volume increase, cells turn from orange to green). In these target cells, both GFP and FRET signals decreased (Fig. 3g, h), however, GFP to a lesser extent compared to the FRET signal, which results in a loss of RFP...
fluorescence and less orange cell appearance (and thus more green). This is also evident by the decrease in donor ratio, indicative of apoptosis. Target cell necrosis and apoptosis are initiated within one minute (= at approximately the same time point, Fig. 3h), and we thus refer to this mode of cell death as a mixed phenotype.

In summary Casper-GR reliably reports a spectrum of target cell death modes following contact with primary human NK cells. Our data also reveal that NK cells kill target cells in multiple ways ranging from apoptosis with or without secondary necrosis via mixed phenotypes to primary necrosis. Interestingly, the very same NK cell can kill one target by primary necrosis and the next one within very few minutes by apoptosis. The target cell death spectrum is not “black and white” with only apoptosis and necrosis, but intermediates exist and primary human NK cells make full use of the spectrum. By a combined quantitative analysis of GFP, FRET and donor ratio, the exact starting point of the cell death mode can be determined.

We used Casper-GR fluorescence changes to create a target cell death plot after NK cell contact for more than 50 cells analyzed from three different blood donors (Fig. 3t). To create the target cell death plot, target cells were randomly chosen with the only exclusion criteria that they did not escape from the field of observation during the entire length of the experiment. The total number of randomly chosen target cells was set to 100% and are presented as a stacked line plot. During long term experiments of 17 hours, NK cells initially kill their targets by both primary necrosis (light grey) or apoptosis (green) while at later times, apoptosis, partially followed by secondary necrosis (dark grey) is predominant. Mixed cell death forms are included in primary necrosis in this analysis. Total killing by either primary necrosis or apoptosis after 17 h is also quantified as mean ± SD of 3 donors (Fig. 3n, o). 68 % of all target cells were apoptotic or secondary necrotic after 17 h. 27% were killed via primary necrosis. Total killing was 95 %, 5 % of target cells survived. Without NK cells almost all targets remain viable over the time of an experiment as also indicated in Fig. 1a.

In cell culture, IL-2 is routinely added to primary NK cells to increase their perforin content and make them more killing competent (28). After 24 hours of IL-2 stimulation, there was a tendency of an increased share of primary necrosis by 15 % to a total of 42 % (Fig. 3j, n), although this difference did not reach statistical significance (p = 0.077). There is, however, a clear increase in the speed of early necrosis induction within the first 2-3 hours. The respective share of apoptotic and secondary necrotic cells was lowered to 58 % (Fig. 3o). Total killing was 100 % (Fig. 3j, p). In conclusion, necrotic killing was enhanced by IL-2 stimulation at the expense of apoptotic killing with the total killing remaining constant over time.

Quantification of perforin/granzyme versus FasL/FasR-induced target cell death by NK cells

NK cells can kill their targets by perforin/granzyme stored in lytic granules or by the FasL/FasR mechanism (14,28). We tested the function of both pathways in four different ways using the single cell death assay:

1. Pharmacological intervention: At low nM concentrations, concanamycin A (CMA) can be used to interfere with the perforin/granzyme pathway without interfering with the FasL/FasR mechanism (14,29). Application of CMA indeed inhibited primary necrosis in target cells induced by NK cells almost completely (down to 2 %) (Fig. 3k, n). Interestingly, the apoptotic portion including secondary necrosis increased to 93 % unmasking FasL/FasR activity following inhibition of the perforin/granzyme pathway (Fig. 3k, o).

Whereas CMA proved to be a very potent inhibitor of perforin based killing, it is more difficult to pharmacologically interfere with FasL/FasR killing. Antibodies against FasL have been shown to partially inhibit FasL toxicity. Using a combination of two clones (NOK-1 and NOK-2) to bind and block two similar but distinct FasL epitopes (30), the apoptotic portion was lowered by 28 % to a total of 30 % apoptotic and secondary necrotic cells in comparison to the IL-2 control (Fig. 3l, o). Thus, as expected, anti-CD178 antibodies could partially reduce apoptosis. Interestingly, the portion of primary necrosis killing was increased by 26 % to 68 %, unmasking the perforin/granzyme activity in case of FasL/FasR inhibition (Fig. 3l, n).

Finally, combining CD178 antibodies and CMA to inhibit both apoptosis and necrosis, primary necrosis was blocked almost completely
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to 2% as by CMA alone (Fig. 3m, n), whereas apoptosis combined with secondary necrosis remained largely unchanged (Fig. 3m, o). Only under these conditions total killing was significantly reduced, otherwise total killing remained the same (Fig. 3p).

2. Induction of apoptosis and necrosis via purified perforin and granzyme B: To elucidate the potential and ratio of necrosis or apoptosis induction by perforin and granzyme B, we tested a concentration series of purified perforin and granzyme B on target cells in the absence of NK cells (Fig. 4).

Without perforin, a concentration of 200 pg/µl granzyme B did not induce apoptosis and all target cells remained viable (Fig. 4a). The addition of increasing perforin concentrations with granzyme B kept at 200 pg/µl led to a dose dependent induction of necrosis (Fig. 4b-d) up to 91% at 200 pg/µl perforin (Fig. 4d) with varying amounts of apoptosis induction, which was highest at 100 pg/µl perforin (Fig. 4c). Perforin alone did only induce necrosis with no apoptosis present (Fig. 4e). We conclude that at high perforin concentrations, target cell necrosis is predominant, probably because target cells cannot repair membrane damage by perforin, while at intermediate concentrations perforin and granzyme B induce also apoptosis probably by the perforin-dependent uptake of granzyme B.

To test if granzyme B may enter the cell in the absence of perforin via endocytosis and induce apoptosis, granzyme B concentration was increased to 1250 pg/µl. At this concentration, granzyme B was able to induce apoptosis in a low number of cells (11%) as indicated at the example in Fig. 4f.

In conclusion, perforin and granzyme B, next to inducing necrosis in a perforin concentration-dependent way, are also able to induce a significant amount of target cell apoptosis and thus very likely contribute to target cell apoptosis induced by NK cells.

3. Lytic granules accumulate at the immune synapse prior to necrosis induction: The complete inhibition of primary necrosis by interfering with perforin release following CMA incubation in combination with the finding that perforin acts exclusively at the immune synapse (Fig. 2f and (27,31)) predict that lytic granules should always be present at the IS in case NK cells kill by necrosis but not necessarily if they kill by apoptosis. To correlate target cell death with NK lytic granule movement, we stained lytic granules with lysoTracker-Red and acquired 3D time lapse data. Lysotracker-Red not only marks lytic granules (and lysosomal compartments) in NK cells but (unfortunately) also in target cells. Therefore, fluorescently-labeled structures in the NK cell are marked by a white arrow to distinguish them from the structures in the target cells, in which the plasma membrane is highlighted with a white line. Fig. S3a shows that the bulk of lytic granules in NK cells is translocated as a group. For every immune synapse analyzed, necrotic target cell death occurred only after the group of lytic granules arrived at the IS, evident by the lysotracker fluorescence signal within the NK cell close to the plasma membrane of the target cell (Fig. S3a, 6 out of 6 cells). To kill several target cells, the group of lytic granules moved sequentially from IS to IS (Fig. S3a) from the first IS formed with the right target to the second one formed with the left target. In case NK cell killed targets by apoptosis, lytic granules were not necessarily relocated to the IS. Fig. S3b shows, that apoptosis of the target is initiated, as indicated by the increased GFP signal, without any lysotracker signal being close to the IS. The statistics confirm that in 50% of apoptosis cases, lytic granules were not localized at the IS, whether in the other 50% they were (n = 8 cells). Thus there is no correlation between target cell apoptosis and lytic granule localization at the IS. This result is in very good agreement with the finding that perforin release from lytic granules is not required for apoptosis (Fig. 3k). However, perforin and granzyme are able to induce apoptosis without FasL/FasR activation (Fig. 4c).

4. Analysis of Fas-deficient target cells: To analyze the role of FasR, K562 pCasper cells which express FasR were compared to Jurkat pCasper cells. We used NK cells from the same blood donor to compare target cell death in both cell lines. Figs. 5a, b confirm what was already shown for other blood donors in Fig. 3: NK cells kill Jurkat pCasper cells both ways, by apoptosis and primary necrosis, whereas in CMA treated Jurkat pCasper cells, primary necrosis is completely inhibited.
The same experiments were carried out with FasR-deficient K562 pCasper targets (Fig. 5c, d). The probability for primary necrosis induced by NK cells was found to be very high, with only very little apoptosis (and secondary necrosis) present. Following incubation with CMA, neither apoptosis nor necrosis of target cells were observed. This confirms that the perforin/granzyme pathway is responsible for all primary necrosis but in addition also initiates apoptosis (and secondary necrosis) in some targets (Fig. 5c) as already suggested by the experiments with purified perforin and granzyme (Fig. 4c).

**Calcium influx changes the apoptosis to necrosis ratio**

Ca\(^{2+}\) entry through Orai channels in NK cells and the concomitant Ca\(^{2+}\) signals are highly relevant for target cell death following immune synapse formation between NK cell and their targets (29,32-34). Using the single cell assay, we tested if Ca\(^{2+}\) entry influences the apoptosis/necrosis ratio. With 1 mM extracellular Ca\(^{2+}\) present, Jurkat pCasper target cells were killed by NK cells similarly as shown before in AIMV medium, which contains 800 µM free Ca\(^{2+}\) (29) with a balanced ratio of apoptosis and primary necrosis, analyzed during 80 min (Fig. 6a). With no external Ca\(^{2+}\) added and remaining Ca\(^{2+}\) buffered by EGTA, primary necrosis was completely inhibited (Fig. 6b) because release of lytic granules and the action of perforin both require Ca\(^{2+}\) (29,33,35,36). Apoptosis was increased under these conditions unmasking the activity of the FasL/FasR pathway.

In case FasR-deficient K562 pCasper cells were used as targets, NK cells killed the target mostly by primary necrosis with very little apoptosis in the presence of 800 µM free Ca\(^{2+}\) in AIMV medium (Fig. 6c), induced by the perforin/granzyme mechanism. However, with drastically reduced external Ca\(^{2+}\) (buffered with 1 mM EGTA, which decreases free Ca\(^{2+}\) in AIMV below 3 µM (29)), both necrosis and apoptosis were basically absent (Fig. 6d), because 1) perforin release at the IS and perforin-mediated target cell lysis both require Ca\(^{2+}\) and 2) FasR is not expressed in K562 cells.

We tested the Ca\(^{2+}\) dependence of the apoptosis-necrosis ratio in more detail. External Ca\(^{2+}\) concentrations between 3 and 4547 µM were applied to the Jurkat pCasper3 target cells and NK cell dependent target cell apoptosis and necrosis were quantified after 2 hours. Whereas apoptosis is more frequent at low Ca\(^{2+}\) (green cells and green bars in Fig. 6e, f) necrosis frequency is significantly increased at higher Ca\(^{2+}\) (grey bars in Fig. 6f). At very low Ca\(^{2+}\), perforin action is impaired (36) and thus necrosis is completely absent below 30 μM. Ca\(^{2+}\) influx largely controls the apoptosis to necrosis ratio.

**Manual versus automated apoptosis and necrosis analysis**

As described in the methods section, we analyzed each cell presented in this work individually and manually confirmed positioning of ROIs and fluorescence values. This analysis method is very reliable but, obviously, time consuming and therefore, we developed a semi-automated analysis routine which can be performed with standard software packages. Target cells were detected using Imaris (Bitplane) and the required mathematical calculations were automated in Excel as described in Materials and Methods.

We compared the results of manual and automated analysis for three blood donors (Fig. S4) using the same experimental conditions as shown in Fig. 3i-m. Between blood donors there are slight differences regarding cytotoxicity of their respective NK cells. The automated analysis matches the manual one qualitatively very well, and in most cases also quantitatively with two exceptions. First, the automated analysis used here does not distinguish between primary and secondary necrosis. Both are counted simply as necrosis but compare well to the sum of primary and secondary necrosis from manual analysis. The second difference is that the onset of necrotic events appears to be slightly earlier in the automated compared to the manual analysis which is most likely attributed to threshold settings.

**Single target cell death analysis in a three dimensional collagen matrix**

One disadvantage working with human cells is that in vivo experiments are not feasible. To mimic physiologically relevant conditions more closely, we established the single cell death assay in a three dimensional (3D) environment. A collagen-matrix based system was established to
analyze interactions between single NK cells and Jurkat pCasper cells in a 3D environment (37). For these measurements, lightsheet microscopy was chosen because it offers very fast measurements of larger three-dimensional samples with minimal dye bleaching and phototoxicity (38). Fig. 7a depicts a reconstructed image of a three-dimensional matrix at one time point obtained by lightsheet microscopy. Target cells are orange (viable) or green (already apoptotic) and NK cells are stained by lysotracker-Red (red colour). In the 3D matrix measurements, target cell apoptosis (Fig. 7b) and primary necrosis (Fig. 7c) following NK cell contact can be easily distinguished and quantified. We performed the same analysis as for 2D measurements. Consistent with caspase dependent target cell apoptosis, the GFP signal increased whereas the FRET slightly decreased and most importantly the donor ratio decreased (Fig. 7d). Consistent with target cell necrosis, fluorescence in the GFP and FRET decreased in parallel whereas the donor ratio remained relatively unchanged (Fig. 7e). These examples show that the single cell apoptosis/necrosis assay can be transferred into a 3D matrix-based environment. The activity of primary human cytotoxic immune cells can be analyzed at a single cell level in a large 3D matrix under well-controlled temperature and pH conditions which together better resemble the physiological in vivo environment than 2D-coated flat surfaces.

We further explored the 3D experimental assay by growing spheroids of MCF-7 cells, a cell line which does not contain caspase-3 (39) but can be killed by NK cell mediated apoptosis (Fig. S5a, b). These experiments also confirm that other caspases cleave the DEVD sequence (21,22). Transfection of caspase-3 into MCF-7 cells, however, had a tendency to accelerate apoptosis (Fig. S5c) indicating that caspase-3 plays an important role following NK cell mediated target cell death. NK cells embedded in a collagen matrix together with MCF-7 spheroids we observed to migrate towards the edge of the spheroid and to induce target cell death as shown in Fig. 7f-h. Thus the assay can be used to monitor target cell death in 3D spheroids.

**Discussion**

The assay developed here allows quantification of target cell death induced through contact with primary human natural killer cells regarding kinetics, modes (apoptosis or necrosis or mixed forms) and mechanisms (perforin/granzyme vs. FasL/FasR) on a single cell level. We found that inhibition of either one of the two major cytotoxic pathways, perforin/granzyme release or FasL/FasR interaction, unmasked the parallel activity of the other one. This may explain why mild perforin malfunction does apparently not cause strong phenotypes but may correlate with long-term cancer development (17).

The prevailing mode of cancer cell death has a large impact on the local tumor environment by shaping the profile of released inflammatory versus tolerogenic substances. For instance, the release of inflammatory substances like ATP (40) is higher in case of necrosis. The possibility to control the apoptosis to necrosis ratio offers an intriguing opportunity to influence the tumor microenvironment. To modify Ca²⁺ influx is one possibility. Ca²⁺ influx through Orai1 channels is important for killer cell function (33-35) and there is an apparent Ca²⁺ optimum for cytotoxicity (29). We found that Ca²⁺ influx regulates the ratio between NK cell induced cancer cell apoptosis and necrosis. An Orai1 channel blocker could thus be an interesting tool to guide the mode of killer cell cytotoxicity by changing the apoptosis to necrosis ratio thereby regulating the immunogenicity of cancer cell death.

Screening cytotoxicity of CTL or NK cells currently receives attention considering the many advances of immunotherapy against cancer (1-4). This is particularly important since human NK cells have recently become more relevant for adoptive cellular immune therapy against cancer (9). Considering the substantial interspecies differences between mice and humans regarding NK cell subsets and receptors, which cause major problems for clinical translation (9), in vitro assays for human NK cells or humanized mice gain relevance. The 2D and 3D assays reported here are ideally suited to analyze the efficiency of natural killer cells of cancer patients including kinetics, mode and mechanism of target cell killing to predict treatment efficiency.

**Experimental procedures**

**Ethical approval**
Research has been approved by the local ethic committee (84/15; Prof. Dr. Rettig-Stürmer). The local blood bank in the Institute of Clinical Hemostaseology and Transfusion Medicine at Saarland University Medical Center provided leukocyte reduction system (LRS) chambers, a byproduct of platelet collection from healthy blood donors. All blood donors provided written consent to use their blood for research purposes.

Reagents, buffers, solutions
Calcein-AM (C3100), and Fura-2/AM (F1211) were purchased from ThermoFisher Scientific. Anti-human CD178 was from BD Biosciences. Anti-human CD95 Apo 1-1 was from Enzo Life Sciences. Mouse IgG1 APC isotype control, mouse IgG1 FITC isotype control and mouse IgG2a PE isotype control were from Biologend. IL-2 was from Invitrogen. Nucleofector Kit V was from Lonza. Vectors for pCasper-GR, TagGFP-N and TagRFP-N were from Evrogen. Bovine serum albumin (BSA) was from Sigma-Aldrich. BSA buffer contained: 2 ml of 1 M HEPES stock, 5 ml of 3 M NaCl stock, ad 50 ml H2O dd, 1 g BSA, pH 7.4, ad 100 ml H2O dd. DPBS was from ThermoFisher Scientific, Dynabeads® Untouched™ Human NK Cells isolation kit was from ThermoFisher Scientific, fetal calf serum (FCS, E.U. approved) was from ThermoFisher Scientific, fibronectin was from Sigma-Aldrich, human granzyme B (ALX-200-602-C010) and perforin (ALX-200-604-C001) were from Enzo, collagen was from AdvancedBiomatrix, llysotrack® Red DND-99 was from ThermoFisher Scientific, PBS was from ThermoFisher Scientific, Penicillin / Streptomycin was from ThermoFisher Scientific, poly-L-ornithine was from Sigma-Aldrich, propidium ioidid was from ThermoFisher Scientific and concanamycin A was from Santa Cruz. RPMI 1640 was from ThermoFisher Scientific, G418 was from ThermoFisher Scientific, staurosorine from Streptomyces sp. was from Sigma-Aldrich and TetraSpeck™ Microspheres were from ThermoFisher Scientific.

0 mM Ca2+ Ringer’s solution contained: 155 mM NaCl, 4.5 mM KCl, 10 mM Glucose, 5 mM Hepes, 3 mM MgCl2, 1 mM EGTA, pH 7.4 (1 N NaOH). 0.5 mM Ca2+ Ringer’s solution contained: 155 mM NaCl, 4.5 mM KCl, 10 mM Glucose, 5 mM Hepes, 2.5 mM MgCl2, 0.5 mM CaCl2, pH 7.4 (1 N NaOH).

All other chemicals and reagents not specifically mentioned were from Merck, J.T. Baker or Sigma Aldrich.

Primary human natural killer (NK) cells
Human peripheral blood mononuclear cells (PBMCs) of healthy donors were isolated from LRS chambers used for thrombocyte apheresis (Institute of Clinical Haematology and Transfusion Medicine, Homburg) by density gradient centrifugation using Lymphocyte Separation Medium 1077 (PromoCell) as previously described (41). Primary natural killer (NK) cells were isolated from PBMC using Dynabead® Untouched™ NK cell isolation kits (ThermoFisher Scientific) according to manufacturer’s specifications as previously described (41). NK cells were incubated at 37 °C, 5 % CO2 in AIM V® media (ThermoFisher Scientific) substituted with 10 % FCS at 2 x 106 cells/ml and, depending on the experiment, either used untreated or substituted with 100 U/ml IL-2 (ThermoFisher Scientific). Purity of NK cells after isolation was checked via flow cytometry analysis. 93.3± 1.6 % of the negatively isolated cells were NK cells, compared to 7.49 ± 1.68 % within the PBMC (mean ± SD of 3 donors). (Fig. S6). Contamination of the purified NK population by Monocytes, B- and T-cells was excluded via CD14, CD19, CD3 and CD4 flow cytometry analysis (Fig. S6). In many publications, NK cells are stimulated by IL-2 (28,42,43). To check the effect of IL-2 stimulation on freshly isolated primary human NK cells, cells were cultivated for 1-8 days in the presence of 100 U/ml IL-2 before use.

Generation of stable target cell lines with Casper-GR
3x10⁶ Jurkat E6-1 (ATCC, TIB-15™) or K562 (ATCC 1994) were transfected with 2 μg pCasper3-GR vector (Evrogen) (20) using a Nucleofector® II (Lonza, program C-016). The transfected population was treated 6 days with 2.5 mg/ml, then 12 days with 1.25 mg/ml G418. Subsequently, the population was seeded in 96 well plates after dilution to 0.5 cells/well. Wells
which contained only one cell were marked and fluorescence signals of growing colonies were assessed. Fluorescent, monoclonal colonies were transferred to bigger flasks. Jurkat E6-1 and K562 cell clones were maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FCS, 1% Penicillin/Streptomycin (Thermo Fisher Scientific) and 1.25 mg/ml G418.

To check the proportion of fluorescent cells, both target cell lines, referred to as Jurkat pCasper and K562 pCasper were analyzed by flow cytometry. Comparing them to the original cell lines, flow cytometry analysis confirmed, that in both cases > 93 % of cells are expressing the fluorescent sensor protein at easily detectable, homogenous levels (Fig. S1).

**2D life cell imaging**

2D life cell imaging was carried out with three different imaging systems: Cell Observer (Zeiss), DMI 6000 B (Leica) or ImageXpress Micro XLS (Molecular Devices). All systems are equipped for 37 °C, 5 % CO₂ incubation.

For the Cell Observer, excitation was realized via Colibri® LEDs 470 nm and neutral white LED with EX BP 560/40. GFP filter set was Zeiss 38 HE (Ex BP 470/40, Em BP 525/50). RFP filter set was 45 HQ (Ex BP 560/40, Em BP 630/75). RFP excitation filter was removed from cube and mounted in front of the neutral white LED to enable GFP excitation to pass the 45 HQ filter cube during FRET experiments.

For the DMI 6000 B (Leica), excitation was realized via a mercury short-arc reflector lamp (EL6000) using Ex 490/20 and Em 525/36 filters for GFP and Ex 552/24 and Em 600/32 filters for RFP.

For the ImageXpress, excitation was realized via Spectra X LED illumination (Lumencor) using LEDs 470/24 and 542/27 for GFP and RFP excitation respectively. The filter sets for FRET were Ex 472/30 and Em 520/35 for GFP and Em 641/75 for RFP. A 20 x S Fluor 0,75NA objective (Nikon) was used.

For imaging, target cells were settled on 25 mm glass coverslips (Kindler) or in black 96 well plates with transparent bottom (Sigma-Aldrich). Both were coated with 50 µl 0.1 ng/ml Fibronectin (Sigma-Aldrich), which facilitates cell migration. Fibronectin was coated onto the coverslip or well bottom for 30 min at room temperature and then aspirated. The coated surface was dried via sterile bench airflow for 10 min and then the target cells (1x10⁵ up to 8x10⁵ cells, depending on desired density) were settled onto the coated surface in cell media for 30 min at 37 °C, 5 % CO₂. Cells were covered with cell media or Ca²⁺ buffer solution, 950 µl in coverslip based microscopy and 100 µl in 96 well plate based microscopy. Either RPMI® or AIM™ were used, both were supplemented with 10 % FCS, 10 mM HEPES and 1 % Pen/Strep for long term experiments. For coverslip-based microscopy experiments, NK cells were added after the acquisition start in a volume of 50 µl, adding up to a total media volume of 1 ml. In 96 well (BD Biosciences) plate-based microscopy, NK cells were added before acquisition start in 100 µl cell media, adding up to a total volume of 200 µl per well. NK cells sank to the coated surface, adhered, started migration and interacted with target cells.

For certain experiments, target cells were stained with calcein (ThermoFisher Scientific), Fura-2 AM (ThermoFisher Scientific) or lysotracker® Red (ThermoFisher Scientific). For calcein or Fura-2 AM staining 5x10⁵ target cells suspended in 1 ml RPMI® + 10 mM Hepes and incubated for 15 min at room temperature on a shaker with 500 nM calcein or 1 µM Fura-2. Cells were centrifuged for 8 min at 300 g, re-suspended in 50 µl RPMI® and settled on a coated coverslip or 96 well plate as described. Fura-2 AM was not imaged ratiometrically but in this case used only as cytosolic label, excited at 360 nm. NK cells were stained with lysotracker® Red by 30 min pre-incubation of 2x10⁵ NK cells in 1 ml AIM™ + FCS + 100 mM lysotracker® Red at 37 °C, 5 % CO₂. NK cells were centrifuged 8 min at 300 g, then re-suspended in 50 µl AIM™ and added to the measurement. For long term measurements, lysotracker® Red was added to the supernatant. To visualize dye influx in target cells following membrane disruption, 100 µM propidium iodide (ThermoFisher Scientific) was added to the supernatant.

**MCF-7 cell culture and transfection**

MCF-7 cells were cultured in MEM medium containing 10% FCS. They were transfected by nucleofection (Amaxa Nucleofector® II, Lonza Kit NHEM Neo, program...
A024) with 2 µg of pcDNA3-Casp3-myc (Addgene plasmid #11813) or 2 µg of empty pcDNA3 vector and 2 µg of pCasper3-GR vector. pcDNA3-Casp3-myc (44) was a gift from Guy Salvesen.

**3D life cell imaging with lightsheet microscopy**

For selective plane illumination microscopy (SPIM) with the Z.1 lightsheet microscope (Zeiss), excitation was realized by two lasers, 488 nm and 561 nm. Emission was filtered via Em 525/40 and Em 585 LP filters.

Nutragen® (Advanced Biomatrix) was used to create 3D collagen matrices after manufacturer specifications. 0.25x10⁶ NK cells were pre-incubated for 30 min with 500 nm lysotracker® Red, mixed with 1.5x10⁶ Jurkat pCasper cells in 50 µl collagen matrix and sucked into a glass capillary (Zeiss). The capillaries were incubated vertically for 1 h at 37 °C, 5 % CO₂ for gelation. For spheroid experiments, MCF-7 cells were transfected as described above and cultured for 24 hours in a low binding 24-well plate (VWR 734-1584) to form spheroids. NK cells and spheroids were mixed in the collagen matrix and incubated horizontally in a capillary for gelation. Imaging was performed in AIM V® substituted with 500 nm lysotracker® red at 37 °C, 5 % CO₂ at 10 or 20 s intervals per stack, depending on the imaged volume.

**Image analysis**

Image analysis and post-processing was done with Axiovision 4.8 (Zeiss), Zen 11.0.0.190 (Zeiss), Image J 1.5i (NIH), Imaris 8.1.2 (Bitplane), Igor Pro 6.2.2.2 (WaveMetrics), Huygens 4.5.1p3 (SVI) and Excel 2010 (Microsoft). Background subtraction, bleaching correction, F/F₀ normalization, donor ratio calculation) were performed with ImageJ and Excel. Using ImageJ, background was subtracted via rolling ball algorithm and cells were tracked manually via Speckle TrackerJ plugin (45). ROI intensities were assessed via Speckle Intensity Trajectories plugin (45). ROI intensity data were processed and visualized via Excel 2010.

**Long term imaging assay (with concanamycin A and anti-CD178)**

The bottom of a black, clear 96 well plate was coated with fibronectin at room temperature and then aspirated. The coated surface was dried via sterile bench airflow for 10 min. Per well, 1x10⁶ Jurkat pCasper target cells in 100 µl AIM V® + 10% FCS + 10 mM HEPES were settled for 30 min at 37 °C, 5 % CO₂. NK cells of different donors were used 1 day after isolation and either used unstimulated or stimulated with 100 U/ml IL-2 (ThermoFisher Scientific). Stimulated NK cells (1x10⁵ per condition) were pre-incubated for 4 h with 200 nM CMA (Santa Cruz) or 20 µg/ml CD178 antibody (BD Biosciences, clones NOK-1 and NOK-2 at 1:1) or both at 37 °C, 5 % CO₂ in a round bottom 96 well plate (BD Biosciences). After pre-incubation, NK cells were added to the targets at 1:1 to a total volume of 200 µl per well, thereby lowering CMA concentration to 100 nM and antibody concentration to 10 µg/ml during the experiment. Total acquisition time was 17 h in 4 min intervals using the ImageXpress Micro XLS (Molecular Devices) at 37 °C, 5 % CO₂. Acquired image data was loaded in ImageJ as time series and background subtraction was performed via rolling ball algorithm.

**FRET evaluation, correction factors, donor ratio calculation**

To evaluate FRET correction factors, Jurkat E6-1 cells were transfected separately with TagGFP-N or TagRFP-N (Evrogen) as described (46). After cultivation, cells were seeded and imaged on coated coverslips as described using the Cell Observer Z1 (Zeiss). Each transfection was checked in donor-, acceptor- and FRET-channel using equal excitation times (300 ms), LED intensities of 25 % and a 20x / 0.75 Fluar objective (Zeiss) (Fig. S7). The percentages of donor bleed through and acceptor crosstalk of the previously described filter setting were determined at 3.6 % and 22.6 % using the FRET and colocalization analyzer plugin for ImageJ (NIH). The corrected FRET signal $F_C$ was calculated by linear unmixing, using the correction factors mentioned as $F_C = (Ch_{det} - BG_{det}) - (Ch_{don} - BG_{don}) * cf_{don} - (Ch_{acc} - BG_{acc}) * cf_{acc}$. Each fluorescent signal (Ch) was corrected by background signals (BG), $cf_{don}$ and $cf_{acc}$ are the slopes of donor bleed through and acceptor crosstalk (Fig. S7). Based on this, the intensity independent donor ratio was calculated by dividing the FRET signal by the background
corrected fluorescence intensity of the donor fluorophore, GFP according to (25) as $F_{\text{DN}} = \frac{F_{\text{ch don}} - F_{\text{BG don}}}{3}$ using the AxioVision physiology module (Zeiss).

**Manual target cell tracking and cell death analysis**

Target cells were tracked manually using the SpeckleTrackerJ plugin for ImageJ. Cells were chosen on a random basis but were discarded in case the left the field of view before the end of the experiment. XY positions for each observed cell were tracked for each time point. Fluorescence intensities were read out using the speckle intensity plugin. Data was imported into Excel, fluorescence values were normalized to the start value of each track and the donor ratio for each cell was calculated, as described above. Judging the fluorescence and donor ratio values as explained in the Results section, the cell status was determined for each time point, being viable, apoptotic, necrotic or secondary necrotic. This information was pooled for all observed cells for each time point. Total number of all observed cells was set to 100%. For each time point the proportion of apoptotic, necrotic or secondary necrotic cells was calculated and displayed in a color coded diagram over time (death plot).

**Automated target cell death analysis**

Target cells were detected using Imaris (Bitplane) but could be done with most available software packages including ImageJ. For each time point cells were detected via spot detection in the GFP fluorescence channel. To avoid tracking errors due to cell collisions and to keep the automated analysis as simple as possible, spots were not connected over time. Therefore secondary necrosis cannot be distinguished from primary necrosis. Fluorescence values for all channels were extracted and data was imported into Excel. For each spot the donor ratio was calculated as described above. Spots with fluorescence values near the background (mean background fluorescence + 10 %) were defined as necrotic (lysed cell debris). Donor ratio and FRET data of all remaining spots (viable and apoptotic) were pooled for each time point. Histograms are shown in Fig. S8. Comparing the donor ratio over time, it is obvious that next to the distribution of living cells at time 0 another population of cells with a donor ratio < 0.6 develops, indicative of apoptosis. Similar as for manual analysis, cells with a clear drop of the FRET signal (in this case below 600 units) are counted as being necrotic. During the very first pictures (before cytotoxicity by NK cells starts), the total number of cells is identical to the total number of detected spots. Unfortunately, necrotic cells can often not be recognized by the spot detection algorithm since their fluorescence is indistinguishable from the background. Therefore, once the number of detected spots decreases due to cytotoxicity, the last detected maximum is kept as the total sum of cells (100 %). This allows us to approximate the proportion of undetected necrotic cells as the number of cells missing to complete the total sum. For each time point the number of viable, apoptotic, or necrotic cells is displayed in a color coded, stacked diagram over time (death plot, Fig. S4).

Automated 3D cell tracking was performed with Imaris (Bitplane) using the GFP fluorescence of target cells with the autoregressive movement model, maximum gap size was 3 time points and maximum movement was 5 μm between time points. Based on this tracking analysis, spherical ROI’s for all fluorescence channels were created. The ROI’s diameter was intentionally set smaller than the real target cell’s diameter to exclude RFP and FRET fluorescence interference from nearby lysotracker® Red-labeled NK cells during synapse formation. For all tracked cells fluorescence intensities were extracted, sorted with IgorPro (Wavemetrics) and processed via Excel.

**Purified perforin (PRF) and granzyme B (GrzB)**

Black, clear bottom 398 well plates (BD Biosciences) were coated with 0.1 mg/ml Poly-L-Ornithine (Sigma-Aldrich) analog to the fibronectin coating. Perforin and granzyme B, purified from YT-NK cells, were obtained from Enzo. Ca²⁺-HEPES buffer and BSA-HEPES buffer were prepared according to the manufacturer’s specifications (Enzo). Per well, 2x10⁵ Jurkat pCasper cells were settled in 20 μl BSA-HEPES. Image acquisition was performed at 37 °C, 5 % CO₂. 15 min after acquisition start, 20 μl BSA-HEPES buffer, containing the desired perforin and granzyme B concentration, were added to each well. 15 min after adding perforin and granzyme...
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B, 40 µl Ca\(^{2+}\)-HEPES buffer were added to each well to induce perforin activation while image acquisition was continued to monitor cell viability. Background correction was performed via rolling ball algorithm in Image J (NIH). Cells were tracked automatically using Imaris (Bitplane). Number and type of cell death were calculated from fluorescence values using Excel (Microsoft).

**Variation of extracellular free Ca\(^{2+}\) concentration in AIMV medium**

AIMV medium with different extracellular free Ca\(^{2+}\) concentrations was prepared as described in (29). The calculated extracellular free Ca\(^{2+}\) concentrations are presented which differ modestly from the ones measured in (29).

**Flow Cytometry**

For details please see Figs. S1, S2 and S5. To check purity of isolated NK cells, cells were stained with an antibody cocktail containing anti-human CD3 (clone SK7) from Biolegend, CD4 (clone RPA-T4), CD14 (clone MoP9), CD16 (clone B73.1), CD19 (clone HIB19), CD45 (clone HI30), C56 (clone NCAM16.2) all from BD Biosciences, washed twice in PBS + 0.5% Jurkat pCasper and K562 pCasper cells were stained with an APC conjugated anti-human CD95 antibody (clone-DX2; Biolegend) for 25 min at RT in the dark in PBS + 0.5% BSA. Data acquisition was performed on a FACSVerse flow Cytometer (BD Biosciences) and analyzed using FlowJo v10.

**Quantitative PCR (qPCR)**

qRT-PCR was carried out with the QuantiTect SYBR® Green PCR kit (Qiagen) using the CFX96™ Real-Time SystemC1000™Thermal Cycler (Software Biorad CFXManager, Version 3.0) as described previously (46). For the expression analysis of CD95 (FasR), the following primer pairs were used: CD95_1_forw: 5'-CAAGGGATTGGAATTGAGGA-3' and CD95_1_rev: 5'-TGG AAG AAA AAT GGG CTT TG-3'; CD95_2_forw: 5'-GTC CAA AAG TGT TAA TGC CCA AGT-3' and CD95_2_rev: 5'-ATG GGC TTT GTC TGT GTA CTC CT-3'. RNAPol and TBP were used as reference genes. Sequences are given in (47).

To change Evrogen’s pCasper-GR base pair at position 1400 from A to C, QuikChange® PCR (Agilent) was used with the following primers: Primer BAN1033 QC caspase cleavage site forward (pCasper: DEVA): 5'-CAG CGG TGA CGA GGT CGC CGG TAC CTC AGT CGC CAC-3'. Primer BAN1034 QC caspase cleavage site reverse (pCasper: DEVA): 5'-GTG GCG ACT GAG GTA CCG GCG ACC TCG TCA CGG CAC-3'. Primers were synthesized by MWG-Biotech.

**Statistical analysis**

Data are presented as mean ± SEM (n = number of experiments) if not stated otherwise. Data were tested for significance using one-way Anova or student's t-test (the latter if Gaussian distribution was confirmed): *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, no significant difference. Statistics were calculated using Microsoft Excel 2010, GraphPad Prism 7.01 and Igor Pro6 (Wavemetrics) software.

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References

1. Kershaw, M. H., Westwood, J. A., and Darcy, P. K. (2013) Gene-engineered T cells for cancer therapy. *Nature reviews. Cancer* **13**, 525-541
2. Smaglo, B. G., Aldeghaither, D., and Weiner, L. M. (2014) The development of immunoconjugates for targeted cancer therapy. *Nature reviews. Clinical oncology* **11**, 637-648
3. Pardoll, D. M. (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nature reviews. Cancer* **12**, 252-264
4. Sharma, P., and Allison, J. P. (2015) The future of immune checkpoint therapy. *Science* **348**, 56-61
5. Weiner, L. M. (2007) Building better magic bullets--improving unconjugated monoclonal antibody therapy for cancer. *Nature reviews. Cancer* **7**, 701-706
6. Thakur, A., Huang, M., and Lum, L. G. (2018) Bispecific antibody based therapeutics: Strengths and challenges. *Blood reviews*
7. Hernandez-Illizaliturri, F. J., Reddy, N., Holkova, B., Ottman, E., and Czuczman, M. S. (2005) Immunomodulatory drug CC-5013 or CC-4047 and rituximab enhance antitumor activity in a severe combined immunodeficient mouse lymphoma model. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 5984-5992
8. Griffin, M. M., and Morley, N. (2013) Rituximab in the treatment of non-Hodgkin's lymphoma--a critical evaluation of randomized controlled trials. *Expert opinion on biological therapy* **13**, 803-811
9. Guillerey, C., Huntington, N. D., and Smyth, M. J. (2016) Targeting natural killer cells in cancer immunotherapy. *Nature immunology* **17**, 1025-1036
10. Sharma, P., and Allison, J. P. (2015) Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell* **161**, 205-214
11. Stinchcombe, J. C., Majorovits, E., Bossi, G., Fuller, S., and Griffiths, G. M. (2006) Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* **443**, 462-465
12. de Saint Basile, G., Menasche, G., and Fischer, A. (2010) Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nature reviews. Immunology* **10**, 568-579
13. Voskoboinik, I., Smyth, M. J., and Trapani, J. A. (2006) Perforin-mediated target-cell death and immune homeostasis. *Nature reviews. Immunology* **6**, 940-952
14. Kataoka, T., Shinohara, N., Takayama, H., Takaku, K., Kondo, S., Yonehara, S., and Nagai, K. (1996) Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *Journal of immunology* **156**, 3678-3686
15. Boudreau, J. E., and Hsu, K. C. (2018) Natural Killer Cell Education and the Response to Infection and Cancer Therapy: Stay Tuned. *Trends in immunology* **39**, 222-239
16. Janka, G. E. (2012) Familial and acquired hemophagocytic lymphohistiocytosis. *Annual review of medicine* **63**, 233-246
17. Voskoboinik, I., Whisstock, J. C., and Trapani, J. A. (2015) Perforin and granzymes: function, dysfunction and human pathology. *Nature reviews. Immunology* **15**, 388-400
NK cells induce distinct modes of cancer cell death

18. Chia, J., Yeo, K. P., Whisstock, J. C., Dunstone, M. A., Trapani, J. A., and Voskoboinik, I. (2009) Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. *Proceedings of the National Academy of Sciences of the United States of America* 106, 9809-9814

19. Imai, K., Matsuyama, S., Miyake, S., Suga, K., and Nakachi, K. (2000) Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 356, 1795-1799

20. Shcherbo, D., Souslova, E. A., Goedhart, J., Chepurnykh, T. V., Gaintzeva, A., Shemiakina, Ii, Gadella, T. W., Lukyanov, S., and Chudakov, D. M. (2009) Practical and reliable FRET/FLIM pair of fluorescent proteins. *BMC biotechnology* 9, 24

21. Benkova, B., Lozanov, V., Ivanov, I. P., and Mitev, V. (2009) Evaluation of recombinant caspase specificity by competitive substrates. *Analytical biochemistry* 394, 68-74

22. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997) Substrate specificities of caspase family proteases. *The Journal of biological chemistry* 272, 9677-9682

23. Caricchio, R., Reap, E. A., and Cohen, P. L. (1998) Fas/Fas ligand interactions are involved in ultraviolet-B-induced human lymphocyte apoptosis. *Journal of immunology* 161, 241-251

24. Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujiwara, T., Roth, J. A., Deisseroth, A. B., Zhang, W. W., Kruzel, E., and et al. (1995) Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Molecular and cellular biology* 15, 3032-3040

25. Youvan, D. C., Silva, C., and Bylina, E. (1997) Calibration of fluorescence resonance energy transfer in microscopy using genetically engineered GFP derivatives on nickel chelating beads. *Biotechnology* 3, 1-18

26. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green, D. R., Hengartner, M., Knight, R. A., Kumar, S., Lipton, S. A., Malorni, W., Nunez, G., Peter, M. E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino, G., and Nomenclature Committee on Cell, D. (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell death and differentiation* 16, 3-11

27. Lopez, J. A., Jenkins, M. R., Rudd-Schmidt, J. A., Brennan, A. J., Danne, J. C., Mannering, S. I., Trapani, J. A., and Voskoboinik, I. (2013) Rapid and unidirectional perforin pore delivery at the cytotoxic immune synapse. *Journal of immunology* 191, 2328-2334

28. Zhu, Y., Huang, B., and Shi, J. (2016) Fas ligand and lytic granule differentially control cytotoxic dynamics of natural killer cell against cancer target. *Oncotarget* 7, 47163-47172

29. Zhou, X., Friedmann, K. S., Lyrmann, H., Zhou, Y., Schoppmeyer, R., Knorck, A., Mang, S., Hoxha, C., Angenentd, A., Backes, C. S., Mangerich, C., Zhao, R., Cappello, S., Schwar, G., Hassig, C., Neef, M., Bufe, B., Zufall, F., Kruse, K., Niemeyer, B. A., Lis, A., Qu, B., Kummerov, C., Schwarz, E. C., and Hoth, M. (2018) A calcium optimum for cytotoxic T lymphocyte and natural killer cell cytotoxicity. *The Journal of physiology*

30. Nisihara, T., Ushio, Y., Higuchi, H., Kayagaki, N, Yamaguchi, N., Soejima, K., Matsuo, S., Maeda, H., Eda, Y., Okumura, K., and Yagita, H. (2001) Humanization and epitope
NK cells induce distinct modes of cancer cell death.

Mapping of neutralizing anti-human Fas ligand monoclonal antibodies: structural insights into Fas/Fas ligand interaction. *Journal of immunology* **167**, 3266-3275

31. Gwalani, L. A., and Orange, J. S. (2018) Single Degranulations in NK Cells Can Mediate Target Cell Killing. *Journal of immunology* doi: 10.4049/jimmunol.1701500

32. Hoth, M. (2016) CRAC channels, calcium, and cancer in light of the driver and passenger concept. *Biochimica et biophysica acta* **1863**, 1408-1417

33. Maul-Pavicic, A., Chiang, S. C., Rensing-Ehl, A., Jessen, B., Fauriat, C., Wood, S. M., Sjoqvist, S., Hufnagel, M., Schulze, I., Bass, T., Schamel, W. W., Fuchs, S., Pircher, H., McCarl, C. A., Mikoshiba, K., Schwarz, K., Feske, S., Bryceson, Y. T., and Ehl, S. (2011) ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 3324-3329

34. Schwarz, E. C., Qu, B., and Hoth, M. (2013) Calcium, cancer and killing: the role of calcium in killing cancer cells by cytotoxic T lymphocytes and natural killer cells. *Biochimica et biophysica acta* **1833**, 1603-1611

35. Lyubchenko, T. A., Wurth, G. A., and Zweifach, A. (2001) Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. *Immunity* **15**, 847-859

36. Schoppmeyer, R., Zhao, R., Hoth, M., and Qu, B. (2018) Light-sheet Microscopy for Three-dimensional Visualization of Human Immune Cells. *Journal of visualized experiments: JoVE*

38. Strobl, F., Schmitz, A., and Stelzer, E. H. K. (2017) Improving your four-dimensional image: traveling through a decade of light-sheet-based fluorescence microscopy research. *Nature Protocols* **12**, 1103

39. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *The Journal of biological chemistry* **273**, 9357-9360

40. Junger, W. G. (2011) Immune cell regulation by autocrine purinergic signalling. *Nature reviews. Immunology* **11**, 201-212

41. Kummerow, C., Schwarz, E. C., Bufe, B., Zufall, F., Hoth, M., and Qu, B. (2014) A simple, economic, time-resolved killing assay. *European journal of immunology* **44**, 1870-1872

42. Clausen, J., Vergeiner, B., Enk, M., Petzer, A. L., Gastl, G., and Gunsilius, E. (2003) Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells. *Immunobiology* **207**, 85-93

43. Frey, M., Packianathan, N. B., Fehninger, T. A., Ross, M. E., Wang, W. C., Stewart, C. C., Caligiuri, M. A., and Evans, S. S. (1998) Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *Journal of immunology* **161**, 400-408

44. Stennicke, H. R., and Salvesen, G. S. (1997) Biochemical characteristics of caspases-3, -6, -7, and -8. *The Journal of biological chemistry* **272**, 25719-25723
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45. Smith, M. B., Karatekin, E., Gohlke, A., Mizuno, H., Watanabe, N., and Vavylonis, D. (2011) Interactive, computer-assisted tracking of speckle trajectories in fluorescence microscopy: application to actin polymerization and membrane fusion. *Biophys J* **101**, 1794-1804

46. Bhat, S. S., Friedmann, K. S., Knorck, A., Hoxha, C., Leidinger, P., Backes, C., Meese, E., Keller, A., Rettig, J., Hoth, M., Qu, B., and Schwarz, E. C. (2016) Syntaxin 8 is required for efficient lytic granule trafficking in cytotoxic T lymphocytes. *Biochimica et biophysica acta* **1863**, 1653-1664

47. Wenning, A. S., Neblung, K., Strauss, B., Wolfs, M. J., Sappok, A., Hoth, M., and Schwarz, E. C. (2011) TRP expression pattern and the functional importance of TRPC3 in primary human T-cells. *Biochimica et biophysica acta* **1813**, 412-423
FOOTNOTES
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The abbreviations used are: NK cells, natural killer cells; immune synapse, IS; lytic granules, LG
Fig. 1

Figure 1. Viability and apoptosis analysis of Jurkat pCasper (Jurkat pC) and K562 pCasper (K562 pC) cells. (a, b) Viability of Jurkat pC and K562 pC cells was analyzed in 96 well plates. Cells were imaged every 5 min in the brightfield, FRET fluorescence of pCasper and GFP signal of pCasper with a 20x objective over 6 h with the cell observer microscope under incubator conditions (37 °C, 5 % CO₂). Spontaneous signs of apoptosis are indicated by the reduction of the FRET signal and corresponding gain
of the GFP signal (green color in the overlay of the brightfield, GFP and FRET channels). Counting dead cells after 6 h revealed that in these conditions > 95% were viable. (c, d) Same experimental setup as in a or b, only that 2.5 µM staurosporine or 5 µg/ml anti-CD95 antibody Apo 1-1 were applied at time 0. (e) Quantification of FRET signals. As in c, 2.5 µM staurosporine was applied to K562 pC cells at time 0. In addition to the brightfield, GFP and FRET overlay as shown in d, FRET-Youvan and FRET donor ratio are calculated (both color coded). Low values are black to dark blue and high ones in red to white. R1 – R4 are marked and turn apoptotic during the experiment. (f) FRET donor ratio kinetics of the cells R1 – R4 from e over 6 h. (g) Quantification of all cells from a-d. Time point of apoptosis induction in each cell was quantified by the abrupt reduction of the FRET donor ratio signal as shown in f. (h) Jurkat cells were transfected with Casper-GR construct with a DEVA mutation in the caspase binding site. Overlay of brightfield, GFP and FRET channel and the FRET donor ratio are depicted for two cells stimulated with 2.5 µM staurosporine or 5 µg/ml anti-CD95 antibody Apo 1-1. Both cells are clearly apoptotic after 2 h as indicated by morphological changes but show no change in fluorescence signals or the FRET donor ratio (h, quantification of n = 10 cells per condition in i).
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Figure 2. Quantification of target cell apoptosis and necrosis by pCasper induced by primary human NK cells. (a) Non-labelled NK cells were applied to Jurkat pCasper target cells loaded in addition with Fura2-AM. Cells were imaged in AIMV medium with the cell observer every 10 seconds and overlays of brightfield + Fura-2 (at 360 nm) or brightfield + GFP + FRET and the FRET donor ratio are shown over time. White arrows mark the two contact sites between one NK cells and two different target cells. (b) Average Fura-2, GFP and FRET fluorescence of 5 target cells from 3 experiments with typical necrotic morphological changes (cell swelling, loss of cytosol and organelles) as the one marked by the white arrow in a at time 0. (c) Average Fura-2, GFP and FRET fluorescence of 5 target cells from 3 experiments with typical apoptotic morphological changes (cell shrinking, blebbing, stop of organelle movement) as the one marked by the white arrow in a at time 5 min. (d) Calcein (500 nM)-loaded Jurkat E6-1 target cells were imaged with the cell observer in 0.5 mM Ca²⁺ Ringer’s solution containing 100 µM PI. Time 0 was set to the starting of fluorescence changes of 1%. One representative killing event is depicted. (e) Average fluorescence of 7 cells from 5 experiments as the one shown in d. (f) Same as d only that calcein fluorescence is not shown and PI is indicated by a color code. One representative killing event is depicted. (g) Relative Fluorescence changes (F/F₀) of the cell shown in f along the dotted line starting from the non-labelled NK cell. Scale bars are 10 µm.
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Figure 3. NK cell contact dependent target cell death plot. Jurkat pCasper cells can be used to distinguish target cell apoptosis, primary necrosis, secondary necrosis (after initial apoptosis) and a mixed phenotype (parallel apoptosis and necrosis) induced by NK cells. Cells are from 7 experiments for a-h. (a) Example of target cell apoptosis (white arrow) as evident by the morphological changes (blebbing, cell shrinking, stop of organelle and cell movement). (b) Time-resolved averaged normalized GFP and FRET fluorescence and the calculated FRET donor ratio for 8 cells. (c) Example of target cell secondary necrosis following an earlier apoptosis as evident from the increased GFP signal (white arrow) evident by the morphological changes (cell swelling, loss of cytosol and organelles). (d) Time-resolved averaged normalized GFP and FRET fluorescence and the calculated FRET donor ratio for 5 cells. (e) Example of target cell primary necrosis (with no evident apoptosis, white arrow) as evident by the morphological changes (cell swelling, loss of cytosol and organelles). (f) Time-resolved averaged normalized GFP and FRET fluorescence and the calculated FRET donor ratio for 6 cells. (g) Example of target cell parallel apoptosis and necrosis (mixed phenotype, necrosis with caspase activity, white arrow) as evident by the morphological changes (blebbing, cell swelling) and fluorescence changes. (h) Time-resolved averaged normalized GFP and FRET fluorescence and the calculated FRET donor ratio for 5 cells. (i-m) Primary NK cells were left untreated or stimulated with 100 units/ml IL-2 for 24h. Cells were settled in a 96 well plate and observed for 17 h with the high content imaging microscope under incubator conditions (37 °C, 5 % CO₂). For each condition 6 experiments (2 experiments from each of 3 blood donors to exclude individual donor effects) were analyzed. Color code for target cells is: viable (orange), apoptosis (green), secondary necrosis after apoptosis (dark grey), primary necrosis including the mixed phenotype (light grey). Time point 0 marks the onset of apoptosis or primary/secondary necrosis, respectively. (i) shows the analysis of 40 NK cells with target cell contacts. (j) 62 NK cells treated with 100 units/ml IL-2. (k) 45 NK cells treated with 100 nM concanamycin A to inhibit necrosis. (l) 52 NK cells treated with 20 µg/ml CD178 antibodies (2 different clones) to inhibit apoptosis, (m) 44 NK cells treated with 100 nM concanamycin A and 20 µg/ml CD178 antibodies (2 different clones) to inhibit necrosis and apoptosis. NK to target cell ratio was 1:1, 10,000 each), about 62,000 cells/cm². Scale bars are 10 µm. (n) Percentage of Jurkat pCasper target cells killed by primary necrosis after 17 h. (o) Percentage of Jurkat pCasper target cells killed by apoptosis and secondary necrosis after 17 h. (p) Percentage of total killing of Jurkat pCasper target cells after 17 h. Data shown as mean ± SD (n = 3 donors). p values were calculated using Ordinary one-way ANOVA.
Figure 4. Induction of apoptosis and necrosis via purified perforin and granzyme B. Target cell death of Jurkat pCasper cells was analyzed following the addition of different combination of recombinant perforin and granzyme B. (a) 0 pg/µl perforin and 200 pg/µl granzyme B (63 cells), (b) 50 pg/µl perforin and 200 pg/µl granzyme B (445 cells), (c) 100 pg/µl perforin and 200 pg/µl granzyme B (516 cells), (d) 200 pg/µl perforin and 200 pg/µl granzyme B (393 cells), (e) 200 pg/µl perforin and 0 pg/µl granzyme B (167 cells). (f) Example with 0 pg/µl perforin and 1250 pg/µl granzyme B. Each experiment was carried out in a single well at incubator conditions in 0 (37 °C, 5% CO₂) in Ca²⁺ free Ringer’s. At -30 min perforin and granzyme B were added and at 0 min 1.25 mM Ca²⁺ was added, which is required for perforin activity. Two time points and the target cell death plot of all cells are shown for each condition. Scale bars are 10 µm.
Figure 5. Ratio of apoptosis and necrosis in FasR expressing compared to FasR-deficient target cells. 75,000 Jurkat pCasper (a, b) or K562 pCasper (c, d) target cells were settled in each well of a 96 well plate and observed for 17 h with the high content imaging microscope under incubator conditions in AIMV medium (37 °C, 5 % CO₂) on a fibronectin coated surface. NK cells (a, c) or NK cells incubated with 100 nM CMA (b, d) from the same blood donor were added at time 0. Target cell death following NK contact was monitored in the brightfield + GFP + FRET overlay and quantified as shown in Fig. 3. For quantification 213 (a), 236 (b), 88 (c) and 143 (d) cells were analyzed out of 4 experiments. Scale bars are 10 µm.
Fig. 6

Figure 6. Calcium influx regulates the apoptosis to necrosis ratio. (a-d) Jurkat pCasper and K562 pCasper were settled on a fibronectin coated coverslip in the presence (a, c) or absence (b, d) of external calcium. NK cells were added at time 0 and killing events were observed for 75 min via brightfield + GFP + FRET overlay. (a) In the presence of external calcium, the FasR positive target cells Jurkat pCasper were killed in a mixture of necrosis, apoptosis and mixed forms via the cumulative effect of perforin, granzymes and FasL/FasR. (b) In the absence of calcium, Jurkat pCasper were only killed via apoptosis due to the perforin’s inability to adhere at the target cell’s membrane. The residual FasL/FasR pathway is able to induce apoptosis in the absence of external calcium. (c) In the presence of external calcium, the FasL deficient K562 pCasper is killed via necrosis, accompanied by some mixed forms caused by perforin mediated granzymes. (d) In the absence of external calcium, K562 pC are not killed at all, since perforin cannot attach to the K562’s membrane nor can apoptosis be induced due to the lack of FasR. (e) During cytotoxicity experiments as outlined in a, the external Ca\(^{2+}\) concentration was varied over a wide range by adding EGTA or CaCl\(_2\) to AIMV. Apoptotic cells are visible by green color. (f) Frequency of...
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apoptotic and necrotic Jurkat pCasper killing as a function of the external free Ca\(^{2+}\) concentration taken from pictures as shown in e two hours after incubation with NK cells. Scale bars are 10 µm.
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Figure 7. Single target cell death analysis in a three dimensional (3D) collagen matrix. (a) Overview of NK cells 3h after starting the experiment (stained by lysotracker-Red, red) and Jurkat pCasper target cells in a collagen matrix imaged by lightsheet microscopy. Some targets are still viable as indicated by the orange fluorescence, some are killed by apoptosis as indicated by green fluorescence (loss of the FRET signal, compare b) and others are killed by necrosis which can be detected by loss of fluorescence (compare c). (b) Example of an individual target cell in the collagen matrix killed by apoptosis following NK cell contact. Contact time between target cell and NK cell was defined as 0 min. The loss of FRET fluorescence after 30 min is evident by the change from orange to green color. (c) Example of an individual target cell in the collagen matrix killed by necrosis following NK cell contact. Contact time between target cell and NK cell was defined as 0 min. The loss of fluorescence after 5 min is evident. (d, e). Kinetics of GFP fluorescence, RFP fluorescence, FRET signals and donor ratios of the cells shown in b and c. (f) MCF-7 cells transfected with pCasper, grown into spheroids and embedded in a collagen matrix together with NK cells, 1h after start of gelation. Maximum intensity projection of pCasper-FRET in red, pCasper-GFP in green, lysotracker-Red also in red. Most target cells express pCasper. Most cells are still viable, but some apoptotic cells can be identified. Some MCF-7 cells show a strong lysotracker signal. (g) NK cells at the border of the spheroid are migrating actively and are identified and marked by their lysotracker signal (depicted in cyan). (h) A representative killing event of an MCF-7 cell is depicted. After contact with the NK cell, the target undergoes shrinking and a change from red to green fluorescence, indicative of apoptosis.
Natural killer cells induce distinct modes of cancer cell death: Discrimination, quantification and modulation of apoptosis, necrosis and mixed forms
Christian S Backes, Kim S Friedmann, Sebastian Mang, Arne Knörck, Markus Hoth and Carsten Kummerow

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