Full title: Live cell evaluation of granzyme delivery and death receptor signaling in tumor cells targeted by human natural killer cells

Running title: Protease-activated reporters of NK function

Alexandra C. Vrazo*, Adrianne E. Hontz*, Sarah K. Figueira*, Braeden L. Butler†, Julie M. Ferrell*, Brock F. Binkowski†, Jinchu Li*, Kimberly A. Risma*§.

*Division of Allergy/Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229. †Promega Corp, Madison, WI 53704. §Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45229.

¶Denotes equal contribution to the manuscript.

Corresponding author:
Kimberly Risma MD PhD
Kimberly.Risma@cchmc.org
Phone: 513-636-7497
Fax: 513-636-3310
Key Points

1. Natural killer cell granzyme B, A and K delivery and subsequent caspase activation is rapid following conjugation with tumor target cells.

2. Natural killer cells also induce caspase activation through death receptor ligation that can be monitored in real time.
Abstract

Growing interest in natural killer (NK) cell-based therapy for treating human cancer has made it imperative to develop new tools to measure early events in cell death. We recently demonstrated that protease-cleavable luciferase biosensors detect granzyme B and pro-apoptotic caspase activation within minutes of target cell recognition by murine cytotoxic lymphocytes. Here, we report successful adaptation of the biosensor technology to assess perforin-dependent and independent induction of death pathways in tumor cells recognized by human natural killer cell lines and primary cells. Cell-cell signaling via both Fc receptors and NK activating receptors led to measurable luciferase signal within 15 minutes. In addition to the previously described aspartase-cleavable biosensors, we report development of granzyme A and granzyme K biosensors, for which no other functional reporters are available. The strength of signaling for granzyme biosensors was dependent upon perforin expression in IL-2-activated NK effectors. Perforin-independent induction of apoptotic caspases was mediated by death receptor ligation and was detectable after 45 minutes of conjugation. Evidence of both FasL and TRAIL-mediated signaling was seen following engagement of Jurkat cells by perforin-deficient human cytotoxic lymphocytes. Although K562 cells have been reported to be insensitive to TRAIL, robust activation of pro-apoptotic caspases by NK cell-derived TRAIL was detectable in K562 cells. These studies highlight the sensitivity of protease-cleaved luciferase biosensors to measure previously undetectable events in live cells in real time. Further development of caspase and granzyme biosensors will allow interrogation of additional features of granzyme activity in live cells including localization, timing, and specificity.
**Introduction**

Natural killer (NK) cells are cytotoxic lymphocytes that provide the first line of defense in the human immune system by recognizing and eliminating tumor cells and virally infected cells. NK cells are reported to execute target cells by a combination of death receptor ligation and secretory granule-mediated killing involving perforin (PRF) and the granzyme family of serine proteases (Grz) \(^1,2\); however, the majority of studies support nearly exclusive usage of the PRF-mediated pathway for rapid cell death. NK cells may recognize target cells directly through a balance of inhibitory and activating receptors, or via coupling of the low affinity IgG receptor, CD16/Fc\(\gamma\)RIIIA, and target cells \(^3\). The latter is termed antibody dependent cellular cytotoxicity (ADCC) and is one pathway utilized by antibody-based immunomodulators, such as rituximab, to eliminate tumor cells.

PRF and Grzs are constitutively expressed by NK cells, although expression can be augmented after cytokine stimulation \(^4-7\). Following NK cell conjugation with a target cell, granules are rapidly exocytosed within minutes \(^8\), and PRF delivers Grzs into the target cell through Ca\(^{2+}\)-dependent oligomerization and pore formation \(^1\). The five human Grzs (A, B, H, K and M) initiate cell death processes through proteolysis of intracellular substrates. GrzB has been shown extensively to activate pro-apoptotic, caspase-dependent pathways through two mechanisms dictated by tumor cell specificity: 1) direct GrzB processing and activation of caspase 3/7, 8 and 10 \(^9-11,12\) and 2) engagement of the mitochondrial pathway to achieve caspase 3/7 activation \(^9\). Much less information is known about the remaining Grzs, largely due to a dearth of functional assays and Grz-specific inhibitors.

Natural killer cells can also kill tumor cells by granule-independent, caspase-dependent mechanisms through death receptor (TRAIL and FAS) ligation \(^13-17\). Following ligation of death receptors on the target cell and initiation of signaling, caspases 8 and/or 10 are reported to activate caspase 3/7 by similar pathways as described for GrzB: 1) direct cleavage of caspase 3 \(^18\) and, in some cells, 2) indirect activation of caspase 3 through the mitochondrial pathway \(^19-21\). The timing and
amplitude of death receptor-induced caspase activation during human NK cell conjugation is not well understood.

Using protease-activated luciferase biosensors expressed in a variety of tumor target cells, we evaluated the kinetics of Grz and pro-apoptotic caspase activation mediated by human NK cell lines and primary human NK cells in real time. We found that activated caspase and GrzB signals were rapidly detected following both Fc receptor (CD16) engagement and direct recognition of tumor target cells by cytokine-activated NK cells. Pro-apoptotic caspase activation was triggered by the secretory granule and death receptor pathways within 30-120 minutes of cell contact. Lastly, we characterized novel tumor cell-based biosensors for GrzA and GrzK, and identified nafamostat mesylate as an inhibitor of GrzK. This study highlights the utility of protease-cleavable biosensors and allows, for the first time, kinetic measurements of granzyme and caspase activity in live NK target cells using a microplate assay.

Materials and Methods

Cells and cell lines

K562 and Jurkat cells were cultured in complete RPMI [supplemented with 10% FBS (Sigma), 100 units/mL penicillin and 100μg/mL streptomycin (Gibco)]. P815 cells were cultured in complete DMEM. NK92 cells were cultured in alpha-MEM (Gibco) with 12.5% equine serum (Hyclone), 12.5% FBS, 0.02mM folic acid, 0.2mM inositol and 200U/mL rIL-2. KHYG1 (from Japanese Collection of Research Bioresources Cell Bank, JCRB0156) were maintained in complete RPMI with 100U/mL rIL-2. The natural killer leukemia (NKL) line was cultured as described 3. Herpesvirus saimiri-transformed human cytotoxic lymphocytes (HVS-CL) were cultured as previously described 22. Human NK cells were obtained from whole blood by negative selection (Miltenyi). To expand NK cells from PBMC without previous selection, 1000U/mL IL-2 was used for 6 d. Human blood
samples were obtained according to protocols approved by our Institutional Review Board, in accordance with the Declaration of Helsinki.

**Reagents**

GLS cAMP reagent (biosensor substrate) was provided by Promega. For luciferase assays, GrzB inhibitor Compound 20 (C20, Synkinase batch YAJB028-035) and pan-caspase inhibitor Q-VD-OPH (MP Biomedicals) were prepared in DMSO. Mg\(^{2+}\)-EGTA was prepared in PBS (3 mM MgCl\(_2\), 4 mM EGTA, pH 7.6). Nafamostat mesylate (Enzo Life Sciences) was prepared in water and used in substrate medium containing heat-inactivated FBS. Anti-human CD16 (3G8), anti-human TRAIL (RIK-2), anti-human FasL (NOK-1) or IgG1k isotype controls (MOPC-21) (All Biolegend) were used in substrate medium. For immunoblots, primary antibodies recognized firefly luciferase (Luci17, Abcam); GrzB (2C5/F5, BD Biosciences); GrzK (H85, Novis Bio); GrzA (GA6, AbD Serotec); perforin (P1-8, Kamiya); and beta-actin (AC-15, Sigma). Secondary antibody was goat anti-mouse IRDye 800 (Licor).

**Biosensor constructs**

Biosensors derived from GloSensor pGLS-30F containing a caspase 3/7 DEVDG cleavage site (hereafter referred to as GLS.DEVD) were constructed as described \(^{23}\) in both the pGLS-30F plasmid and a retroviral vector pMIEG expressing GFP in \(cis\) \(^{24}\). pGLS-30F plasmids containing Grz A/K-specific sites \(^{25,26}\) were generated using the following oligonucleotides: GLS.SGR, forward: 5’-GATCCTCGGGCCGTAGCGGA-3’, and reverse: 5’-AGCTTCCGCTACGGCCCGAG-3’; GLS.PGPR, forward: 5’-GATCCCCGGGGCCCAGGGAGGGA-3’, and reverse: 5’-AGCTTCCCTCCCTGGGCCCCCGGG-3’; GLS.QGPR, forward: 5’-AGCTTCCCTCCCTGGGCCCCCGGG-3’, and reverse: 5’-AGCTTCCCTCCCTGGGCCCCCGGG-3’. For the sequences of control plasmids, please contact the authors.
In vitro testing of recombinant biosensors

Recombinant luciferase protein was generated and tested as described \(^{23,27}\) with recombinant human GrzB (ImmunoChemistry), GrzA (Millipore) or GrzK (Abcam). To inhibit GrzA and GrzK activity, 3.5\(\mu\)M nafamostate mesylate was pre-incubated with 0.42U/\(\mu\)L enzyme for 15 min at 37°C. Fold activation was expressed as: (Signal\(._{\text{enzyme}}\)/Signal\(._{\text{no enzyme}}\)). Percentage inhibition of signal was expressed as: [1-(Signal\(._{\text{inhibitor}}\)/Signal\(._{\text{diluent}}\)]\(\times100\).

Transfection and stable transduction of cell lines

Clonal and polyclonal cell lines expressing GLS biosensors were obtained as previously described.\(^{23}\) KHYG1 cells were transduced with retrovirus to overexpress miR30-based shRNAs targeting the 3’UTR of PRF1 with mCherry expressed in cis\(^{28}\). Transduced KHYG1 cells (KHYG1 shPRF) were sorted for mCherry expression 7 d later.

Biosensor assay

The assay was performed as described with target cells at 20,000 cells per well in quadruplicate\(^{23}\). RLU were measured every 3 min in a GloMax-Multi+ luminometer set at 37°C (0.5 s integration time). Fold activation for each timepoint represent four replicate wells and was calculated using: (RLU\(_{\text{E+T}}\)/RLU\(_{\text{T alone}}\)). Statistical analysis by one-way ANOVA and Dunnett’s multiple comparison test was performed in GraphPad Prism v.6.0f.

\(^{51}\)Cr-release assay

A \(^{51}\)Cr assay was performed as previously described\(^{23}\). Target cells were incubated with \(^{51}\)Cr for 1 h, then effector and target cells (5,000 cells/well) were co-incubated for 4 h at 37°C. Cytotoxicity is reported as the percentage of \(^{51}\)Cr release into supernatant, calculated as follows: [(test -
spontaneous) / (maximum lysis - spontaneous)] ×100. Maximum lysis was determined by addition of 1% Triton X-100 to 5,000 target cells.

**Immunoblots**

Cell lysates were prepared with lysis buffer (2% NP-40; 150mM NaCl; 50mM TrisCl (tris(hydroxymethyl)aminomethane chloride); 1mM MgCl₂, pH 8; 2.5mM EDTA, (ethylenediaminetetraacetic acid), 2μg/mL leupeptin; 2μg/mL aprotinin; 50μg/mL PMSF; 1μg/mL pepstatin; 10μM Q-VD-OPH; 50μM C20). Following standard immunoblotting procedures, nitrocellulose membranes (Millipore) were visualized with a LICOR Odyssey CLx scanner. Relative expression of perforin protein in KHYG1 shPRF to KHYG1 was calculated as follows: [(shPRF density PRF / density actin) / (KHYG1 density PRF / density actin)].

**Flow cytometry**

Surface staining was performed with antibodies specific to human CD3, CD8, CD56, CD16 and CD95/Fas (BD). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD) as per the manufacturer’s protocol, following staining with antibodies specific to perforin (clone δG9, Biolegend) and granzyme B (Invitrogen). Data was acquired on a BD FACSCanto II with BD FACSDIVA software (V8.0.1) and analyzed on FlowJo X (V10.0.7).

**Results**

**Protease cleavable biosensors are activated by recombinant human granzymes**

We have previously demonstrated murine GrzB activation of luciferase biosensors containing three cleavage sequences (GLS.IETD, -IEAD, and -VGPD)²³. Based on cleavage prediction by GraBCas, we hypothesized that the biosensors would be activated by human GrzB (Table 1). Luciferase was
generated by *in vitro* translation and cleavage was tested with recombinant human GrzB (Fig 1A). Cleavage and activation of luciferase biosensors was dependent on the concentration of recombinant human GrzB (Fig 1A). Confirming previous findings, the GLS.DEVD biosensor and three additional control biosensors with a non-cleavable alanine at P1 could not be activated by GrzB (Fig 1B).

As GrzA is one of the most abundant granzymes expressed by human NK, we developed three new luciferase biosensors containing proteolytic sequences identified to be cleaved by GrzA; GLS.SGR, GLS.PGPR, and GLS.QGPR (Table 1). Recombinant human GrzA activated both GLS.SGR and GLS.PGPR, with GLS.SGR demonstrating the largest fold activation (Fig 1C). As GrzK has also been reported to recognize arginine in the P1 position, we also evaluated cleavage by this tryptase. Recombinant human GrzK activated GLS.SGR but not GLS.PGPR (Fig 1D). Neither enzyme could activate GLS.QGPR (Fig 1C and 1D). In addition, recombinant GrzB was unable to activate the GrzA/K sensors GLS.SGR or GLS.PGPR (Fig 1E). Based on reports of GrzA inhibition, we reasoned that the pan-tryptase inhibitor, nafamostat mesylate (NM), would inhibit both GrzA and GrzK-induced luciferase activity. Following pre-incubation with recombinant GrzA and GrzK, NM inhibited biosensor activation induced by both enzymes, although the magnitude of GrzA inhibition was greater than GrzK (Fig 1F). While subtle, these findings represent the first report of GrzK inhibition by nafamostat mesylate.

*NK stimulation by CD16 delivers granzyme B and activates pro-apoptotic caspases in target cells*

We previously reported that anti-CD3 triggered degranulation of herpesvirus saimiri (HVS)-transformed human cytotoxic lymphocytes (HVS-CL). Degranulation led to activation of the caspase 3/7 biosensor (GLS.DEVD) expressed by P815 target cells, a murine tumor line that binds murine-derived antibodies. Unfortunately, HVS-CL do not reliably express the NK Fc receptor.
CD16. Therefore, to evaluate Fc receptor-mediated NK function, we evaluated CD16/ FcγRIIIA expression by flow cytometry on NK92, KHYG1, and NKL human cell lines and, consistent with previous publications\(^{3,37,38}\), determined that only NKL cells expressed CD16 (Supp Fig 1A). We also confirmed that the NK effectors expressed PRF and GrzB by flow cytometry (Supp Fig 1B). Using a redirected killing assay as a surrogate for ADCC, we conjugated NK effector cells with anti-CD16 and P815 target cells expressing GLS.DEVD or GLS.VGPD. Luminescence was measured in live cells every 3 min for 240 min on a plate reader warmed to 37°C. Figure 2A shows that NKL cells were capable of robustly activating the GLS.DEVD biosensors in P815 cells, similar to our previous findings with anti-CD3 stimulation\(^{23}\). The caspase-mediated luciferase signal was rapid, peaking at 60 minutes, followed by a slow decline over the 240 min time course. We also measured CD16-mediated granzyme B delivery by NKL cells using P815 cells expressing GLS.VGPD and noted a similar pattern of rapid activation (Fig 2A). In contrast, KHYG1 cells that lacked surface CD16 exhibited no biosensor activation (Fig 2B), despite expressing PRF and GrzB (Supp Fig 1B).

*Granzyme and pro-apoptotic caspase biosensors are activated through direct NK cell recognition of tumor target cells.*

Using primary murine NK cells cultured in IL2, we previously demonstrated modest activation of GLS.DEVD and GLS.VGPD in YAC cells, a murine NK target\(^{23}\). To expand our studies to human NK, we transduced Jurkat and K562 cells with retroviruses expressing the GLS.DEVD, -VGPD, -IETD or -IEAD biosensors and sorted for GFP-positive cells (Supp Fig 2A). We next evaluated direct NK-mediated killing using NK92, KHYG1 and NKL cells as effectors. The most robust signal in both target cells was mediated by NK92 cells, the cell line which expressed the highest levels of PRF and GrzB\(^{39,40}\) (Supp Fig 1B). NK92 cells rapidly activated all four biosensors within minutes in Jurkat and K562 (Fig 3A and 3B). The maximal fold induction differed between the target cells and
was not predicted by the relative amount of retroviral expression (Supp Fig 2A). Jurkat cells demonstrated preferential activation of the GLS.IETD sensor. In K562 cells, the GLS.VGPD generated the highest fold activation in signal. Variation in peak biosensor signal was also observed between experiments; for example, GLS.VGPD ranged from 16-80 fold in K562, and from 4-10 fold in Jurkat. For this reason, one representative experiment of several is graphed. Due to the optimal, rapid signals obtained using K562 versus Jurkat target cells, we focused the majority of NK experiments using K562 target cells.

We assessed the dependence of the luciferase signal on GrzB and/or caspase activity by pre-treating cells with a pan-caspase inhibitor (Q-VD-OPH) \textsuperscript{41} or a selective human GrzB inhibitor (C20) \textsuperscript{42}. Based on cleavage prediction by GraBCas that the VGPD site would be restricted to granzyme B proteolysis, we expected the GLS.VGPD signal to be suppressed by the inhibitor of GrzB, but not the pan-caspase inhibitor. This is clearly illustrated in Fig 3C where there is no apparent impact of Q-VD-OPH on the NK92-mediated signal, and near complete suppression with C20 using K562 target cells expressing GLS.VGPD. Using this same concentration of C20, we measured caspase 3/7 activation in K562 cells. We anticipated that C20 would also fully inhibit the GLS.DEVD biosensor. As expected, Q-VD-OPH ablated the GLS.DEVD signal (Fig 3D). Surprisingly, C20 only partially blocked caspase 3/7 activation; a late signal was consistently detected in K562 expressing GLS.DEVD (Fig 3D). The most likely explanation for this finding is that the GLS.DEVD signal resulted from residual GrzB activity; however, we could not exclude the alternate hypothesis that GLS.DEVD activation was mediated via death receptor activation.

*Novel granzyme A/K biosensor is activated through direct NK cell recognition of tumor target cells*

We next tested the capacity of the newly developed GrzA/K biosensors to be triggered in the same kinetic live cell assay. Since GLS.SGR gave the highest signal following *in vitro* cleavage with both GrzA and GrzK, K562 cells were transfected with GLS.SGR, and a clonal line (GLS.SGR.5) that
expressed the highest levels of luciferase was selected in order to maximize the GLS.SGR signal (Supp Fig 2B). NK92 cells were conjugated at an E:T of 3:1 with K562 cells to maximize the modest GLS.SGR signal (Fig 3E). Activation of the GLS.SGR biosensor was secretory granule-dependent, as Mg²⁺-EGTA eliminated the signal. We tested the specificity of activation of GLS.SGR by performing assays in the presence of NM, C20 or Q-VD-OPH. The GLS.SGR signal was inhibited only by NM, indicating that GrzB and caspases do not contribute to GLS.SGR activation (Fig 3F).

**Human primary NK cells require IL-2 activation to activate granzyme biosensors**

Like NK cell lines, ex vivo human NK cells are cytotoxic, constitutively express perforin and granzymes, and exhibit enhanced function upon IL-2 stimulation. We hypothesized that primary NK could induce biosensor activation and that IL-2 stimulation would further enhance biosensor signal. Following isolation of primary human NK cells (>60% CD3⁻ CD56⁺) from peripheral blood mononuclear cells (PBMC), we measured NK cytolysis of K562 cells by Cr51 release assay. Function was nearly comparable to NK92 cells and significantly enhanced following culture in IL-2 for 24 h (Fig 4A).

Next, we assessed the ability of primary NK to activate GrzB and caspase 3/7 biosensors, using either retrovirally-transduced K562 cells, or K562 clonal cell lines derived to express the highest level of biosensor signal. As shown in Figure 4B, primary human NK and IL-2-stimulated NK were unable to activate the GrzB biosensor, GLS.VGPD, expressed in K562 clone 1 (Fig 4B, Left), suggesting that 24 h IL-2 stimulation is insufficient to upregulate adequate GrzB. However, primary NK elicited minimal activation of the caspase 3/7 biosensor (GLS.DEVD) in K562 clone 6 by 90 min, and this signal was amplified following IL-2 stimulation (Fig 4B, Right). To further enhance the biosensor signal, we increased the E:T of the conjugation assay to 3:1 and extended the assay to 240 min. However, we were unable to detect activation of GLS.VGPD or GLS.SGR.
Minimal detection of late GLS.DEVD activation after 90 min was again observed, similar to Fig 4B. The data obtained using retrovirally transduced, polyclonal K562 cells was identical; no GLS.VGPD or GLS.SGR biosensor signal was noted in the absence of IL-2 stimulation, and minimal activation of GLS.DEVD (data not shown).

NK cell function was also tested using over 20 individual donor PBMC at an E:T of 20:1 (equivalent of NK E:T of 0.25-2:1, depending upon donor). In the absence of IL-2 stimulation, a measurable signal was never obtained using K562 expressing GLS.VGPD or GLS.SGR. Occasional samples showed a minimal, late GLS.DEVD signal. We cultured NK from PBMC using the same donors shown in Figure 4C in 1000 units IL-2 for 6 d, and conjugated effectors at 3:1 with K562 clonal lines. Figure 4D indicates that this alternate expansion NK cell protocol led to a robust biosensor signal for GLS.VGPD and GLS.DEVD. The magnitude of increase of GLS.SGR signal was less dramatic, yet also approached the modest signal induced by NK92 cells.

*Granzyme B and caspase biosensor activity correlates with NK cell perforin content*

To evaluate PRF dependence of the NK kinetic biosensor assay, we silenced PRF protein expression in the KHYG1 NK cell line using a shRNA expressed by a retroviral vector. KHYG1 was chosen as this line gave reproducibly higher retroviral transduction levels compared to other NK effectors. PRF protein was reduced by >80% as confirmed by immunoblot (Fig 5A) and PRF-deficient KHYG1 cells demonstrated reduced cytolytic ability (Fig 5B). Analogous to the results obtained by pharmacologic inhibition of GrzB, inhibition of PRF expression in KHYG1 cells suppressed activation of all 4 biosensors in K562 cells. Figure 5 shows the K562 lines with the highest signal were the GrzB sensor, GLS.VGPD (Fig 5C), and GrzB/caspase 8 sensor, GLS.IETD (Fig 5D). As the PRF knockdown in KHYG1 shPRF was not 100%, it is possible that some of the GrzB biosensor activation could be due to residual GrzB delivery.
As previous reports indicated the majority of HVS-CL exhibited NK-like activity against K562 and Jurkat tumor target cells\textsuperscript{22,47}, we tested the capacity for PRF-sufficient (PRF\^{+\+}) and PRF-deficient (PRF\^{\textasciitilde}) HVS-CL to activate the GrzB and caspase biosensors in K562 cells. The PRF\^{\textasciitilde} cells were derived from a patient with hemophagocytic lymphohistiocytosis with no PRF protein (Supp Fig 3A, B) due to biallelic 50delT truncations in \textit{PRF1}\textsuperscript{23}. Following 1:1 conjugation with biosensor-expressing K562 cells, PRF\^{\textasciitilde} HVS-CL did not activate GLS.VGPD or GLS.IEAD compared with PRF\^{+/+} HVS-CL (Fig 5E-F). Despite being unable to kill K562 target cells in a cytolytic assay (Supp Fig 3C), PRF\^{\textasciitilde} cells activated the GLS.IETD (GrzB/caspase 8) and GLS.DEVD (caspase 3/7) biosensors at a slightly delayed onset and a significantly lower level (Fig 5G-H). We previously showed that PRF\^{\textasciitilde} HVS-CL activate the caspase 3/7 biosensor in P815 cells through Fas activation\textsuperscript{23} but K562 cells do not express Fas (Supp Fig 2C)\textsuperscript{48,49}. Taken together, these data suggested that the caspase 3/7 (GLS.DEVD) and caspase 8 biosensors (GLS.IETD) were activated by PRF and Fas-independent mechanisms during receptor-mediated direct recognition of K562 by HVS-CL.

\textit{Perforin-deficient human cytotoxic lymphocytes activate pro-apoptotic caspases in tumor target cells by death receptor ligation}

PRF-deficient murine NK cells can kill target cells by activating death receptor pathways\textsuperscript{23}, but similar findings using PRF-deficient human NK cells have not been described. K562 and Jurkat cells both express TRAIL receptors, DR4/DR5, but only Jurkat cells are reported to be sensitive to rapid onset, TRAIL-mediated death\textsuperscript{50,51}. Likewise, Fas is also expressed by Jurkat,\textsuperscript{48,49} and reports have indicated killing by NK92.\textsuperscript{52} To measure the effect of death receptor function on caspase biosensor activation in K562 cells, we incubated PRF\^{\textasciitilde} HVS-CL with blocking antibodies against TRAIL prior to and during conjugation with target cells. Surprisingly, TRAIL blockade completely inhibited caspase 3/7, 8 and 10 biosensor activation in K562 cells (Fig 6A and B). To our knowledge, this is
the first report of early caspase activation in K562 cells through NK cell-mediated TRAIL ligation. We repeated these experiments with PRF−/− HVS-CL and Jurkat cells, focusing on both TRAIL and Fas. Figure 6C indicates that blockade of TRAIL ligation led to incomplete diminution of caspase 8 biosensor (GLS.IETD) activation in Jurkat cells. Blocking FasL did not reliably inhibit the GLS.IETD signal unless TRAIL was concurrently blocked, demonstrating that both FasL and TRAIL death receptors were activated on Jurkat target cells by PRF-deficient NK cells (Fig 6C-D). Taken together, these results indicate that caspase activation in the context of PRF-deficient NK cells is dependent on death receptor ligation, and highlights differing sensitivities of tumor cell lines to caspase activation following death receptor signaling.

**DISCUSSION**

Although the five human Grz activate distinct methods of caspase-dependent and -independent cell death in tumor cells and cytotoxicity is initiated rapidly by human NK cells, techniques to study kinetic Grz and caspase activation during target cell death are limited. Recent developments in murine systems now permit examination of the kinetics of Grz and caspase activation during CTL-mediated apoptosis, and visualization of tumoricidal caspase 3/7 activity in animals. Here, we have used luciferase biosensors to measure GrzB, GrzA, GrzK and pro-apoptotic caspase activation in real time following human NK cell cytotoxicity induced by CD16 ligation and NK receptor-mediated recognition.

Our study highlights the differences in biosensor magnitude between two different tumor target cell lines, K562 and Jurkat, despite similar vector expression levels (Supp Fig 2). For example, K562-expressed caspase 3/7 biosensors exhibited far superior magnitude compared to P815 (Fig 2) and Jurkat biosensors (Fig 3). In addition, the activity of GLS.IETD was robust in Jurkat cells in comparison to the other Jurkat biosensors (Fig 3A). Whether the differences in biosensor signal
between P815, K562, and Jurkat cells are ascribable to cell-specific Grz inhibitor expression, death receptor activity, luciferase stability, ATP content, or another mechanism, is currently unknown.

The flexibility of the luciferase technology allowed the development of biosensors to measure the kinetics of delivery of GrzA and GrzK by human NK cells, and subsequent characterization of GrzA and GrzK inhibitors. Using the GLS.SGR biosensor at an E:T of 3:1, we detected delivery and activation of GrzA/K within 30 min, analogous to GrzB activation. The signal generated by GLS.SGR was modest compared to the other biosensors, which was surprising despite the higher E:T used when evaluating its activity in K562 cells, and the abundance of GrzA, GrzK and PRF in NK92 cells (Supp Fig 1C). The low signal could be explained by differences in accessibility of the enzyme active site or proteolytic sequence due to steric hindrance. It has also been suggested that GrzA accumulates in the target cell nucleus, rather than the cytoplasm where the biosensors are expressed.

The identification of nafamostat mesylate (NM) as an inhibitor of NK cell-derived GrzA and GrzK is an important finding. NM was previously shown to be a GrzA inhibitor in vitro and to partially inhibit T cell-mediated cytolysis. Several reports indicate that NM reduces cytotoxic lymphocyte-mediated target cell lysis without identifying the mechanism of inhibition. Despite evidence from murine models and a study demonstrating decreased NK92 cytotoxicity following GrzK knockdown, the importance of GrzA and GrzK to the cytotoxic capacity of human NK is unclear. Studies with combinations of NM and other protease inhibitors may begin to address these questions.

The magnitude of GrzB and pro-apoptotic caspase biosensor activation in K562 cells was largely dependent on GrzB and PRF delivery, as we found that IL-2-activated NK cell lines expressing high levels of PRF and GrzB induced rapid, robust biosensor activation, compared with NK cells expressing lower concentrations of cytotoxic proteins. As PRF and GrzB are constitutively expressed in resting, primary human NK, we were surprised by the absence of GrzA and GrzB.
biosensors activation at 3:1 E:T using K562 clones with high level luciferase expression (Fig 4B and 4C). While it is possible that the biosensors lack sensitivity to detect the cytoplasmic granzyme signal, an alternate possibility is that primary NK cells do not deliver sufficient granzymes to the cytoplasm to cleave the protease-activated luciferase. In addition, it was surprising that GLS.DEVD biosensor activation was minimal and delayed in response to primary NK (Fig 4C). Whether the late caspase 3/7 activation elicited by primary NK is due to GrzB activity below the level of biosensor detection, or due to death receptor ligation, is under further investigation.

We evaluated primary NK cell function using K562 cells rather than Jurkat or P815 due to the enhanced signal:noise found with K562 cells, and the reported absence of death receptor activity.\textsuperscript{50,51} Future studies will focus on evaluating whether primary NK cells may activate pro-apoptotic caspases by death receptor activation in more susceptible cell lines, such as Jurkat or P815.

We previously identified that PRF-deficient HVS-CL use FasL to trigger activate caspase 3/7 in P815 cells.\textsuperscript{23} In the current study, we found that caspase activation in Jurkat was also partially dependent on Fas ligation (Fig 6), confirming previous reports.\textsuperscript{61,62} The highest signal induced by PRF deficient cells was with caspase 8 sensor, GLS.IETD (Fig 6), in both K562 and Jurkat, confirming that caspase 8 is the first initiator caspase activated by DR4/DR5 and Fas receptor ligation in these tumor lines.\textsuperscript{18} In contrast, the signal from caspase 10 biosensor, GLS.IEAD, was lower in magnitude. This may relate to suboptimal luciferase function or reflect the limited role reported for caspase 10 in death receptor signaling in these cells.\textsuperscript{21}

The finding of significant caspase 3/7 activation mediated by TRAIL in K562 cells was quite surprising as previous reports noted the resistance of this cell line to TRAIL-induced cell death at 24 hours.\textsuperscript{63,64} Our studies indicate that early TRAIL signaling may lead to low level of caspase 3/7 activation in K562 (Fig 6B) with an insufficient signal to bring about cell death. Future studies will be required to determine if the caspase signal induced by PRF-deficient cytotoxic lymphocytes is limited to robust activation of a small fraction of the tumor cells whose death is not detectable by
cytolytic assays, or is due to activation of pro-apoptotic caspases at an insufficient magnitude to induce cell death.

In summary, the current study presents target cell-expressed biosensors as powerful tools to interrogate differences in cytotoxic function of human NK cells, and to allow further delineation of PRF and death-receptor mediated pathways. The ability to create and define novel GrzA and GrzK luciferase biosensors demonstrates the possibility of adaptation of the technology to understanding the kinetics of other granzymes involved in NK cell killing. In addition, using this novel methodology, we have revealed previously undetectable events in TRAIL-mediated early caspase activation in K562 cells, opening the door for future studies exploring the resistance to death in this myeloid leukemia cell line.
Authorship
A.V. designed and performed research, analyzed data, and wrote the paper; A.H. designed and performed research, analyzed data, and revised the paper; S.F. designed and performed research, analyzed data, and revised the paper; B.L.B. designed and performed research and analyzed data; J.F. and J.L. designed research, analyzed data, and revised the paper; B.F.B. designed research and revised the paper; K.R. designed research, analyzed data and revised the paper.

Conflict of interest disclosure
Brock Binkowski and Braeden L. Butler declare competing financial interests as employees of Promega. The remaining authors declare no conflict of interest.

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References


Figure Legends

Figure 1. Protease cleavable biosensors are activated by recombinant human granzymes

Luciferase was expressed by in vitro translation and activation was tested following cleavage with recombinant human granzyme B (A, B, E), granzyme A (C) or granzyme K (D) over different concentrations. Recombinant granzyme A or K (0.42U/uL) were pre-incubated with nafamostat mesylate before addition to GLS.SGR (granzyme A, K) or GLS.PGPR (granzyme A) (F). Following 1 hr of incubation of in vitro translated luciferase with enzyme, luciferase reagent was added, RLU were measured, and fold activation or percentage inhibition was calculated as described in Methods. Error bars indicate SEM for three experiments.
Figure 2. Granzyme B delivery and caspase 3/7 activation are detected during redirected NK killing.

Human NK cell lines activate caspase 3/7 and granzyme B biosensors in P815 tumor target cells during redirected killing, as a model for ADCC. P815 cells expressing GLS.DEVD and GLS.VGPD were pre-incubated with anti-CD16 antibody or isotype control for 30 min at 37°C before incubation with NK effector cells at 1:1 (20,000 target cells/well). Cells were spun into contact and RLU was measured in a luminometer set at 37°C every 3 min for 240 min, and every 6 min is plotted for clarity. Each datum point represents the mean of four replicate wells. Shown is a representative figure from three independent experiments using two effector cells lines: (A) NKL cells; (B) KHYG1 cells.
Figure 3. Biosensors are activated following direct NK cell recognition of tumor target cells.

Caspase and granzyme biosensors are activated in tumor target cells following NK effector conjugation. NK92 cells were conjugated at 1:1 with each target cell line expressing biosensors and RLU was measured every 3 min for 240 min. The mean datum point at every 6 min is plotted. Fold activation in signal is shown for (A) Jurkat cells and (B) K562 cells. For inhibitor studies, K562 cells were pre-incubated with Q-VD-OPH (10uM) or diluent and NK92 cells were pre-incubated with C20 (50uM), then fold activation in signal was measured for: (C) K562-GLS.VGPD and (D) K562-GLS.DEVD. (E) NK92 cells were conjugated at 3:1 with a K562 clonal line (GLS.SGR.5) expressing the GrzA/K sensor with diluent (PBS) or Mg^{2+}-EGTA in PBS (4mM). (F) To test specificity of GLS.SGR, K562 cells were pre-incubated with Q-VD-OPH (10uM) or diluent, and NK92 cells were pre-incubated with C20 (50uM), nafamostat mesylate (NM, 3.5uM) or diluent, before conjugation at 3:1. Only NM inhibited GLS.SGR activation (P<0.0001). The fold change in signal from three independent reads at 96 min is indicated. Data are represented as mean ± SD. ***, P<0.001; ns, not significant by one way ANOVA. Shown is a representative figure from at least three independent experiments.
Figure 4. Primary human NK cells elicit limited biosensor activation without IL-2 stimulation.

Primary and IL-2-activated human NK cells were assessed for biosensor activation with K562 biosensors at an E:T of 3:1. Human NK cells were negatively selected from healthy donor PBMC by magnetic bead isolation and confirmed to be >60% CD3⁻CD56⁺ by flow cytometry. NK92 was used as a positive control. (A) NK and 24 hr IL-2 stimulated NK are cytotoxic against K562 targets. Error bars represent SD from quadruplicate wells. One representative experiment of two is shown. (B) NK and 24 hr IL-2 stimulated NK are unable to activate the GLS.VGPD sensor in K562 clonal lines at an E:T of 0.75:1 (Left), although GLS.DEVD activation (Right) was observed by 90 min following stimulation with IL2. One representative experiment of three with and without IL2 stimulation is shown. (C) To maximize the biosensor signal, isolated NK were conjugated at a higher E:T (3:1) and the read extended to 240 min. NK cells were unable to activate GLS.VGPD (Left) and GLS.SGR (Middle), although consistent late activation of GLS.DEVD (>90 min) was observed (Right). (D) Following 6 d culture in 1000 U/ml IL-2, NK expanded from PBMC readily activated GLS.VGPD (Left), GLS.SGR (Middle) and GLS.DEVD (Right) expressed in K562 clonal lines at 3:1. The fold activation for one representative experiment of 3 is shown.
Figure 5. Caspase biosensors are activated by perforin-dependent and -independent mechanisms.

The perforin dependence of the biosensor signal was tested with shRNA silencing of PRF1 in KHYG1 cells (A-D) and a patient-derived line deficient in PRF (E-H). (A) Expression of PRF in parental KHYG1 line and in KHYG1 shPRF transduced with shRNA specific to PRF1 3’UTR. PRF expression relative to KHYG1 is shown. Relative PRF expression to KHYG1 is shown below. (B) Cytotoxicity of KHYG1 shPRF cells against K562 targets is decreased. Error bars represent SEM from >10 independent experiments. Luciferase biosensors were sensitive to PRF knockdown in KHYG1 shPRF cells following 1:1 conjugation with K562 cells expressing (C) GLS.VGPD or (D) GLS.IETD. (E-H) HVS-CL line from PRF-deficient patient (PRF−/−) or PRF-sufficient donor (PRF+/+) reveals late caspase activation following 1:1 conjugation with K562 expressing: (E) GLS.VGPD, (F) GLS.IEAD, (G) GLS.IETD or (H) GLS.DEVD. For luciferase assays, each datum point represents the mean of four replicate wells. One representative graph is shown from three independent experiments with similar results.
Figure 6. Perforin-deficient NK cells activate caspase biosensors by Fas and TRAIL death receptor ligation.

HVS-CL cells from a perforin-deficient patient (PRF−/−) were pre-incubated with blocking antibodies or with isotype control antibodies, then incubated with tumor target cells in a 4 hr luciferase assay at 1:1. Fold signal activation over target cells alone was calculated. (A, C) Representative time course of signal activation in target cells expressing GLS.IETD caspase 8 biosensor. Each datum point represents the mean of four replicate wells. (B, D) The fold change in signal from three independent reads at 240 min was normalized to the isotype control. Data are represented as mean ± SEM from three experiments. (A, B) K562 target cells. (C, D) Jurkat target cells. **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant by one way ANOVA.
Tables and Figures

Table 1. Sequence and cleavage sites of granzyme and caspase biosensors.

<table>
<thead>
<tr>
<th>Sensor name</th>
<th>Predicted cleavage site</th>
<th>Protease(s) predicted to cleave site</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLS.IEAD</td>
<td>SGRIEADSESL</td>
<td>Granzyme B; Caspase 10</td>
</tr>
<tr>
<td>GLS.IETD</td>
<td>SGIETDSGSL</td>
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<td>Caspase 3/7</td>
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<tr>
<td>GLS.SGR</td>
<td>GSSGRSGSL</td>
<td>Granzyme A; Granzyme K</td>
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<td>GLS.PGPR</td>
<td>GSPGPRGSL</td>
<td>Granzyme A; Granzyme K</td>
</tr>
<tr>
<td>GLS.QGPR</td>
<td>GSQGPRGSL</td>
<td>Granzyme A; Granzyme K</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A) NKL cells
CD16+

- GLS.VGPD + αCD16
- GLS.DEVD + αCD16
- GLS.VGPD + isotype
- GLS.DEVD + isotype

B) KHYG1 cells
CD16-

- GLS.VGPD + αCD16
- GLS.DEVD + αCD16
- GLS.VGPD + isotype
- GLS.DEVD + isotype
Live cell evaluation of granzyme delivery and death receptor signaling in tumor cells targeted by human natural killer cells

Alexandra C. Vrazo, Adrianne E. Hontz, Sarah K. Figueira, Braeden L. Butler, Julie M. Ferrell, Brock F. Binkowski, Jinzhu Li and Kimberly A. Risma