Membrane-Bound TRAIL Supplements Natural Killer Cell Cytotoxicity Against Neuroblastoma Cells

Michael A. Sheard*, Shahab Asgharzadeh†,‡, Yin Liu*, Tsen-Yin Lin†, Hong-Wei Wu*, Lingyun Ji†, Susan Groshen‡, Dean A. Lee§, and Robert C. Seeger*,†,‡
*Division of Hematology/Oncology, Children’s Hospital Los Angeles (CHLA), Los Angeles, CA, USA
†Keck School of Medicine, University of Southern California, Los Angeles, CA
‡Children’s Oncology Group, Arcadia, CA
§Division of Pediatrics, MD Anderson Cancer Center, University of Texas, Houston, TX

Abstract

Neuroblastoma cells have been reported to be resistant to death induced by soluble, recombinant forms of TRAIL (CD253/TNFSF10) due to low or absent expression of caspase-8 and/or TRAIL-receptor 2 (TRAIL-R2/DR5/CD262/TNFRSF10b). However, their sensitivity to membrane-bound TRAIL on natural killer (NK) cells is not known. Comparing microarray gene expression and response to NK cell-mediated cytotoxicity, we observed a correlation between TRAIL-R2 expression and the sensitivity of fourteen neuroblastoma cell lines to the cytotoxicity of NK cells activated with IL-2 plus IL-15. Even though most NK cytotoxicity was dependent upon perforin, the cytotoxicity was supplemented by TRAIL in fourteen of seventeen (82%) neuroblastoma cell lines as demonstrated using an anti-TRAIL neutralizing antibody. Similarly, a recently developed NK cell expansion system employing IL-2 plus lethally irradiated K562 feeder cells constitutively expressing membrane-bound IL-21 (K562 clone 9.mbIL21) resulted in activated NK cells derived from normal healthy donors and neuroblastoma patients that also utilized TRAIL to supplement cytotoxicity. Exogenous IFNγ up-regulated expression of caspase-8 in three of four neuroblastoma cell lines and increased the contribution of TRAIL to NK cytotoxicity against two of the three lines; however, relatively little inhibition of cytotoxicity was observed when activated NK cells were treated with an anti-IFNγ neutralizing antibody. Constraining the binding of anti-TRAIL neutralizing antibody to membrane-bound TRAIL but not soluble TRAIL indicated that membrane-bound TRAIL alone was responsible for essentially all of the supplemental cytotoxicity. Together, these findings support a role for membrane-bound TRAIL in the cytotoxicity of NK cells against neuroblastoma cells.
INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is expressed both as a soluble, secreted protein and as a surface membrane-bound protein. In TRAIL-susceptible cells, ligation of TRAIL-receptors can lead to activation of caspase-8 and subsequently caspase-3 to induce apoptosis.\textsuperscript{1–3} In resistant cells, ligation of TRAIL-receptors by soluble TRAIL has been shown to stimulate proliferation, at least partly through activation of the NF-κB and MAPK pathways.\textsuperscript{4, 5} TRAIL can be expressed by multiple cell types, including activated T lymphocytes and activated natural killer (NK) cells.\textsuperscript{6}

Neuroblastoma is an embryonal tumor that originates from neural crest-derived precursor cells. Human neuroblastoma cells lack basal expression of surface human leukocyte antigen (HLA) class I and II molecules, and are therefore likely to avoid recognition by host T lymphocytes.\textsuperscript{7–9} Unlike T cells, NK cells are activated by cells lacking HLA molecules, and can therefore kill neuroblastoma cells.\textsuperscript{10, 11} In neuroblastoma patients, NK cell activity is believed to be required for therapeutic efficacy following clinical administration of an antibody against disialoganglioside (GD2) expressed on neuroblastoma cells.\textsuperscript{12}

Activated natural killer (aNK) cells attack tumor cells using two direct cytotoxic pathways. In the first pathway, target-directed exocytosis of lytic granules releases perforin at the immunological synapse to form pores in the target cell membrane, allowing co-secreted granzymes to enter target cells and activate the caspase cascade. In the second pathway, ligation of TRAIL, CD95-ligand (Fas-ligand, CD178), or tumor necrosis factor-α (TNF-α) to their cognate receptors on target cells induces cell death in sensitive or sensitized target cells.\textsuperscript{2, 13–16} In contrast with TRAIL and CD95-ligand, TNF-α can induce inflammation rather than cell death in many cell types, dependent at least in part on the ubiquitination status of RIP1.\textsuperscript{2} In addition to these pathways, activated NK cells are also known to produce IFNγ which is required for NK cell function against multiple cancer types.\textsuperscript{14–16}

While most human neuroblastoma cell lines express TRAIL-receptor 2 (TRAIL-R2), as a group they are generally insensitive to induction of apoptosis by soluble, recombinant TRAIL or agonistic anti-TRAIL-R2 antibodies.\textsuperscript{17–20} This resistance of human neuroblastomas to soluble TRAIL is, in many cases, related to their low expression of caspase-8 and caspase-10 which lie downstream of TRAIL-R1 and -R2 signaling. Caspase-8 expression is suppressed by gene methylation in most human neuroblastoma cell lines,\textsuperscript{17–19} and in about half of clinical neuroblastomas,\textsuperscript{21} as well as in some other pediatric tumor types.\textsuperscript{21} In neuroblastoma cells, previous findings indicate that caspase-8 may be the chief arbitrator of cell death downstream of cross-linking of soluble TRAIL, when anti-Flag antibody was used to cross-link recombinant Flag-TRAIL.\textsuperscript{22} In a large panel of neuroblastoma cell lines that contained a subset expressing basal caspase-8 and caspase-10, caspase-10 expression paralleled that of caspase-8 but was several-fold lower, and incomplete knock-down of caspase-8 in TRAIL-sensitive neuroblastoma cell lines rendered...
the cells resistant to apoptosis induced by cross-linked TRAIL, regardless of their endogenous expression of caspase-10. This suggested that the relatively low expression of endogenous caspase-10 was not sufficient to initiate apoptosis, even in the presence of a low level of caspase-8 expression.22

Given that the majority of neuroblastoma cells are reported to be resistant or to proliferate in response to soluble TRAIL,4, 5, 17–20 we determined whether membrane-bound TRAIL on NK cells contributes to cytotoxic activity against neuroblastoma cells.

MATERIALS AND METHODS

Blood samples

Written informed consent was obtained directly from anonymized healthy adult donors in accordance with a protocol approved by the Committee on Clinical Investigation (CCI) at Children’s Hospital Los Angeles for the use of cells for cancer and/or blood research. A second protocol was approved by the CCI to obtain anonymized specimens from patients with high-risk, stage 4 (metastatic) neuroblastoma enrolled and consented in therapeutic and/or biology protocols of the Children’s Oncology Group (COG).

Cell lines and reagents

Culture media were supplemented with 10% fetal bovine serum, penicillin, and streptomycin. SMS-SAN, SMS-KCN, SMS-KCNR, SMS-KAN, SMS-KANR, SK-N-BE(2), LA-N-1, LA-N-2, and LA-N-6 human neuroblastoma cell lines, as well as HeLa cervical carcinoma cells and K562 clone 9.mbIL21 feeder cells were maintained in RPMI-1640 medium. The K562 clone 9.mbIL21 cell line was made by transducing the human acute myeloid leukemia cell line K562 with several constructs, including genes that encode a membrane-bound form of interleukin-21 (IL-21) and CD137L.23, 24 CHLA-15, CHLA-20, CHLA-42, CHLA-51, CHLA-79, CHLA-90, CHLA-134, and CHLA-136 human neuroblastoma cell lines were established at our institute and maintained in their original medium, Iscove’s Modified Dulbecco’s Medium. All neuroblastoma cell lines expressed GD2 (data not shown) and passage numbers below 30 were used when available. Cell culturing was performed at 37° C in humidified 5% CO2. IL-2 was from Peprotech Inc. (Rocky Hill, NJ). IL-15 was kindly provided by Amgen (Thousand Oaks, CA). Human serum was from Omega Scientific (Tarzana, CA). Human FcR blocking agent was from Miltenyi Biotec (Cambridge, MA). Concanamycin A (CMA) was from Sigma-Aldrich (St. Louis, MO). IFNγ was from R&D Systems (Minneapolis, MN). Recombinant soluble TRAIL was from Enzo Life Sciences (Farmingdale, NY). A list of all antibodies used in this study is presented in Supplemental Table 1.

Gene expression profiling by oligonucleotide microarray analysis

Total RNA was isolated and processed on U133A and U133B microarray chips from Affymetrix (Santa Clara, CA), as previously described.25 Together, the U133A and U133B gene chips contained 45,000 probe sets representing approximately 33,000 transcripts. TRAIL-R2 probe sets reside on the U133A chip, and the 209295_at probe set was used to detect TRAIL-R2 because it provided the strongest correlation with flow cytometry-defined
surface protein expression. The fluorescence values (fluorescent units, F.U.) of probe sets were normalized using the invariant probe set method and modeled using the perfect match minus mismatch algorithm as implemented in the dChip program. Gene expression values obtained from these sets were log(base 10) transformed before analysis. Data was analyzed with dChip and Genetrix (Epicenter Software, Pasadena, CA) software, as described.

**Immunoblotting**—A total of $10^7$ cells were harvested and lysed in RIPA buffer (Upstate, Charlottesville, VA) for protein extraction. Twenty µg of protein per lane was fractionated on 8% Tris-Glycine gels (Invitrogen, San Diego, CA), transferred to nitrocellulose membranes, probed with primary antibodies, and incubated with the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were treated with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 2 minutes and visualized on autoradiography film (Denville Scientific, Metuchen, NJ).

**Enrichment of fresh NK cells by magnetic cell sorting (MACS)**—Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood of human volunteers using histopaque gradient separation (Sigma-Aldrich, Milwaukee, WI). NK cells were enriched by depleting non-NK cells using the MACS column provided in the NK cell negative isolation kit from Miltenyi Biotec (Auburn, CA), using the manufacturer’s instructions. The purity of each cell preparation was confirmed using flow cytometry by staining for CD56$^+$ CD3$^-$ cells, and when less than 90%, cells were re-run through a fresh column to increase purity and again checked by flow cytometry.

**Flow cytometry**—For polychromatic analysis of surface protein expression on human PBMC, cells were washed in FACS buffer (PBS, 0.1% FBS, 0.1% NaN$_3$), Fc-receptors were blocked for 15 minutes using FcR blocking agent, and cells were incubated with a pre-mixed antibody cocktail for 90 minutes at room temperature in the dark, followed by two washes. For two-color analyses of neuroblastoma cells, cells were detached using Puck’s EDTA, washed in ice-cold FACS buffer and incubated with antibody for 45 minutes at 4°C in the dark, washed twice, and filtered through a 40 µm mesh to remove clumps. DAPI (0.5 ng/ml final concentration) was added to all samples, and data was acquired on a four-laser LSRII (BD Biosciences) using a U.V. laser to excite DAPI. During data analysis, dead cells and debris were excluded according to their increased staining with DAPI and low forward scatter properties, respectively. For polychromatic analyses, compensation for the optical spillover of fluoresced light into unintended channels was performed using antibody capture beads (anti-mouse kappa chain; BD Biosciences, San Diego, CA) incubated with the same antibodies as used in the experimental tubes, and the AutoComp software routine was employed to calculate the appropriate amount of compensation (DIVA software version 6.0, BD Biosciences). Histogram overlays were prepared using FlowJo software (TreeStar Inc., Ashland, OR). In polychromatic and two-color experiments, 300,000 events and a minimum of 30,000 events, respectively, were acquired for each sample. Mean fluorescence intensity (MFI) Index was calculated as follows: (MFI of viable cells stained with specific antibody) / (MFI of viable cells stained with an isotype-matched irrelevant antibody).

_J Immunother. Author manuscript; available in PMC 2014 June 20._
Expansion and activation of fresh NK cells—To expand and activate NK cells with IL-2 plus IL-15, fresh NK cells were isolated from PBMC using MACS, and isolated NK cells were passaged every 4-5 days, with each passage including IL-2 (40 ng/ml) alone in the first week and then with the addition of IL-15 (10 ng/ml) in subsequent weeks. aNK cells were used in functional assays at 3-5 weeks after initial isolation. For NK cells expanded using feeder cells, PBMC were incubated with lethally irradiated (100 Gy γ-rays) K562 clone 9.mbIL21 cells (2:1 ratio) plus low-dose IL-2 (5 ng/ml, ~ 50 IU/ml) and re-fed in the same manner on day 7 according to a previously described protocol.23, 24

In vitro cell-mediated cytotoxicity assays—Tumor cells were loaded with the fluorescent dye calcein-AM (5 µg/ml final concentration) for 30 minutes at 37° C, washed, and then 5x10^3 cells/well were plated in 96 well plates with or without 5x10^4 non-labeled aNK cells. Calcein-AM fluorescence intensity is directly proportional to the number of viable cells. Cytotoxicity was measured within 12 hours of loading calcein-AM, to avoid the non-specific leakage of calcein-AM from cells that occurs after longer incubations. aNK cell-mediated cytotoxicity against neuroblastoma targets after an 8- or 12-hour co-incubation (specified in the figure legends) was measured as the loss of fluorescence from target cells, and quantified on a custom built fluorescence-based digital image microscopy system (DimScan),27, 28 available from BioImaging Solutions, Inc. (San Diego, CA). Results were expressed as Percent Tumor Cell Survival, calculated as follows: [(fluorescence of labeled neuroblastoma cells in co-cultures with non-labeled aNK cells) / (fluorescence of labeled neuroblastoma cells alone)×100].

In vitro proliferation assay—Cell lines were detached using Puck’s EDTA solution, pipetted vigorously to obtain single cell suspensions, and seeded in 96-well plates as 2,000 cells in 200 µl of medium per well, 10 wells per condition. Cells were treated with soluble TRAIL immediately after the seeding of each plate, and plates were then cultured for 96 hours. Before acquiring data, plates were gently loaded using a multichannel pipettor with 50 µl/well of a staining solution containing ten parts of a 1% eosin Y solution (to quench background fluorescence), nine parts of an isotonic NaCl solution, and one part of a 200 µg/ml fluorescein diacetate solution (solubilized in DMSO). Plates were incubated in the dark for 30 minutes, and then relative cell numbers were measured by fluorescence-based digital image microscopy, as described above.27, 28 Results were expressed as Relative Change in Cell Number (%), calculated as follows: [(fluorescence of neuroblastoma cells treated with soluble TRAIL) / (fluorescence of untreated neuroblastoma cells)×100].

Statistics—The associations between gene expression and sensitivity to aNK cells, between expression of surface protein and sensitivity to aNK cells, and between gene expression and expression of surface protein were summarized using the Spearman correlation coefficient. Effects of pretreatment of aNK cells with anti-TRAIL, anti-CD95L, and anti-TNF-α neutralizing antibodies on aNK cell-mediated cytotoxicity were investigated using mixed-effect Analysis of Variance (ANOVA). These were considered fixed effects, as were presence or absence of cell-conditioned medium and the interaction between cell-conditioned medium and the antibodies for the experiment that examined whether the effect of anti-TRAIL neutralizing antibody was due to blocking soluble or membrane bound
TRAIL. Other effects, such as when multiple plates were used, were considered to be random effects. Pair-wise comparisons were performed using the least significant difference method once the overall F-test was significant at $\alpha = 0.05$. Fluorescence readings from the in vitro cytotoxicity assays were assumed to have a lognormal distribution, and were transformed to the natural log scale before analyses were conducted. All p values reported were two-sided. STATA software version 11.2 was used.

**RESULTS**

**TRAIL-R2 expression associates with neuroblastoma cell sensitivity to aNK cell-mediated cytotoxicity**

To identify gene products associated with sensitivity to aNK killing, an 8-hour calcein-AM in vitro aNK cytotoxicity assay was used to determine the sensitivity of a panel of neuroblastoma cell lines to cytotoxicity by NK cells that were expanded and activated by IL-2 plus IL-15 for three weeks. Results were compared with gene expression profiles obtained from oligonucleotide microarray analysis of the same cell lines. No correlation was observed between tumor cell survival from aNK killing and mRNA expression of FADD, Bid, caspase-8, -3 or other caspases (data not shown); however, the level of mRNA expression of TRAIL-R2 in tumor cells was inversely correlated with tumor cell survival in aNK cytotoxicity assays (Spearman correlation coefficient = -0.60, p = 0.023) (Fig. 1A). An inverse association was also observed between surface protein expression of TRAIL-R2 and tumor cell survival (Spearman correlation coefficient = -0.55, p = 0.022) (Fig. 1B). Data from two cell lines, SMS-KAN and CHLA-134, did not fit with the inverse association, indicating that mechanisms independent of TRAIL-R2 can regulate neuroblastoma cell resistance to NK cytotoxicity. Notably, the expression of TRAIL-R2 surface protein and mRNA correlated well with each other (Spearman correlation coefficient = 0.62, p = 0.019) (Fig. 1C), demonstrating the validity of the oligonucleotide microarray probe for TRAIL-R2 mRNA. These findings suggested that TRAIL-R2 expression level might be a contributing factor to neuroblastoma sensitivity to aNK cytotoxicity.

As TRAIL-R2 is not the only receptor for TRAIL, we evaluated other members of the TRAIL-receptor family. The cell lines in our panel uniformly exhibited little or no TRAIL-R1 surface expression, whereas HeLa cells expressed a relatively high level (Fig. 1D). Surface expression of decoy receptors TRAIL-R3 and TRAIL-R4 was variable and no correlation with sensitivity to aNK cell-mediated cytotoxicity was observed (data not shown).

**IL-2- plus IL-15-expanded NK cells use TRAIL to supplement perforin/granzyme-mediated cytotoxicity against neuroblastoma cells**

A differential response to membrane-bound versus soluble ligands of the TNF super family has been reported previously. To determine the effect of membrane-bound TRAIL, we expanded and activated human NK cells with IL-2 plus IL-15 for three weeks. This activation induced membrane-bound TRAIL from virtually no surface expression on non-activated cells to relatively high levels on expanded cells (data not shown, and see below), as previously reported. Pre-incubation of aNK cells with an anti-TRAIL neutralizing

*J Immunother. Author manuscript; available in PMC 2014 June 20.*
antibody was used to block TRAIL signaling in aNK cell-mediated cytotoxicity assays. When data from the seventeen cell line panel were analyzed together, the overall survival effect of pretreatment of aNK cells with anti-TRAIL neutralizing antibody was significantly different compared to aNK alone (p < 0.001). Separate analysis of data on each cell line demonstrated that fourteen of seventeen (82%) cell lines reproducibly exhibited a statistically significant increase in survival after pretreatment of aNK cells with anti-TRAIL neutralizing antibody, at α = 0.05 (Fig. 2A). Of the five cell lines expressing the least amount of TRAIL-R2 protein (Fig. 1B), anti-TRAIL neutralizing antibody had no effect in three lines and only a slight effect in the other two (Fig. 2A), consistent with a previous report that expression level of TRAIL-R2 is one determinant of sensitivity to TRAIL. Across all 17 cell lines in the panel, an average 1.5-fold increase (95% confidence interval (CI) = 1.42 – 1.59, p < 0.001) was observed in neuroblastoma cell survival between those co-incubated with untreated aNK cells versus aNK cells pretreated with anti-TRAIL neutralizing antibody. Concerning the effect of neutralizing antibodies against other death ligands at this 8-hour time-point, only a slight effect was observed on aNK cell-mediated cytotoxicity and only in some cell lines when aNK cells were pretreated with anti-TNF-α neutralizing antibody (an average 1.14-fold increase when all replicates of all cell lines were evaluated together, 95% CI = 1.08 – 1.21, p < 0.001), and no significant difference was observed across all cell lines following pretreatment of aNK cells with anti-CD95L (p = 0.35). As expected, the perforin pathway was critically required for cytotoxicity, as the exocytosis inhibitor concanamycin A (CMA) prevented nearly all target cell death of all cell lines in the panel. The observations above suggested that TRAIL expression on aNK cells can supplement cytotoxicity against most neuroblastoma cell lines.

We next examined the effect of soluble recombinant TRAIL on our panel of cell lines. A total of 6 of 17 (35%) cell lines exhibited increased proliferation in response to soluble TRAIL. In 2 of those 6 lines (SMS-KCNR and SMS-KAN), a dose effect pattern of proliferation was induced by soluble TRAIL (Fig. 2B). Unexpectedly, in two other lines (SMS-KCN and SK-N-BE(2)) all concentrations induced proliferation, suggesting that even the lowest dose examined might have some effect in those cells. In the final two lines (SMS-SAN and LAN-6), an increase was observed at intermediate concentrations but not the highest concentration. This latter result is consistent with a previous report that a high concentration of soluble TRAIL can simultaneously induce both proliferation and apoptosis in some cell lines, whereas lower concentrations induce only proliferation in such lines. Notably, no statistically significant reduction in cell number was observed in any cell line at any concentration of soluble TRAIL. Interestingly, two of the cell lines (SK-N-BE(2) and LAN-6) that exhibited a proliferative response to soluble TRAIL (Fig. 2B) were not sensitive to the cytotoxic effect of membrane-bound TRAIL (Fig. 2A), consistent with the separate signaling pathways thought to regulate these divergent responses.

K562 clone 9.mbIL21-expanded NK cells use TRAIL to supplement cytotoxicity against neuroblastoma cells

Recently, the antigen-presenting K562 clone 9.mbIL21 feeder cell system for expanding and activating NK cells from PBMC was developed and characterized for potential clinical application in adoptive cell therapy. This system involves the ex vivo co-culturing of
PBMC with irradiated K562 feeder cells engineered to express membrane-bound IL-21 and CD137L, plus the addition of exogenous, soluble IL-2. We examined the relative contributions of feeder cells and IL-2 to cell surface expression of CD56, CD16, and TRAIL on aNK cells expanded with this system (Fig. 3A). NK cells (CD56<sub>low</sub> CD16<sup>+</sup> and CD56<sub>high</sub> CD16<sup>neg</sup>) from freshly thawed, non-incubated PBMC obtained from normal donors did not express TRAIL (Fig. 3A, first column). Incubation of the same PBMC for fourteen days in RPMI-1640 medium with 10% fetal bovine serum resulted in a minor population of NK cells expressing a low level of TRAIL, as well as a major population of CD3<sup>+</sup> T lymphocytes expressing no TRAIL (Fig. 3A, second column). Treatment of the PBMC for fourteen days with IL-2 alone (5 ng/ml) resulted in a minor population of NK cells expressing high levels of CD56 and TRAIL (Fig. 3A, third column). Co-incubation of the PBMC with feeder cells alone resulted in a major population of NK cells expressing an intermediate level of CD56 and a low level of TRAIL (Fig. 3A, fourth column). However, only the combined treatment of PBMC with feeder cells and IL-2 resulted in a major population of NK cells that expressed high levels of CD56 and TRAIL (Fig. 3A, fifth column).

Although TRAIL expression on aNK cells treated with IL-2 alone or IL-2 plus feeder cells was similar at the end of fourteen days, total aNK cell expansion was not. After adjusting for the frequencies of the NK cell subset at the time of initial seeding and at the end of the two week incubation, cell counting indicated a 12-fold increase in viable NK cell numbers during the fourteen day incubation with IL-2 alone, a 420-fold increase with feeder cells alone, and a 2,460-fold increase with feeder cells plus IL-2, in the representative experiment shown (Fig. 3B). These findings show that the K562 clone 9.mbIL21 feeder cell system plus IL-2 expands and activates large numbers of NK cells that express a high level of TRAIL.

To examine whether K562 clone 9.mbIL21-expanded aNK cells utilize TRAIL during cytotoxicity, NK cells from four healthy donors and four neuroblastoma patients were expanded (Fig. 4). These aNK cells were not treated or treated with neutralizing antibodies against death ligands as in Figure 2 and then co-cultured with calcein AM-labeled SMS-KAN and CHLA-134 neuroblastoma cells for twelve hours. Although some donor variability was observed in the amount of aNK cell-mediated cytotoxicity, TRAIL was found to supplement cytotoxicity regardless of donor (Fig. 4).

NK cells are known to produce both membrane-bound and soluble TRAIL. We examined five aNK cell preparations from healthy donors and five from neuroblastoma patients, both expanded using the K562 clone 9.mbIL21 feeder cell system, and found that soluble TRAIL was detectable at concentrations of 46 (+/- 19) pg/ml and 22 (+/- 15) pg/ml, respectively (manuscript submitted for publication). To examine the relative role of membrane-bound TRAIL in supplementing aNK cell cytotoxicity, the binding of neutralizing anti-TRAIL antibody was constrained exclusively to membrane-bound TRAIL by the following method. Expanded aNK cells from healthy donors were thawed, allowed to recover for three days in culture with IL-2 (5 ng/ml), cells were pelleted, and their culture media (supernatants) were set aside as conditioned medium (CM). Next, the aNK cells were washed again and then pretreated with anti-TRAIL neutralizing antibody for one hour. Subsequently, all media were carefully aspirated during two consecutive washing steps to remove excess antibody,
and then aNK cells were re-supplied with either fresh medium or with their own CM (75% final concentration) prior to inclusion in cytotoxicity assays, such that only membrane-bound TRAIL was neutralized. Constraining the binding of anti-TRAIL neutralizing antibody solely to membrane-bound TRAIL inhibited aNK cell cytotoxicity even in the presence of re-supplied NK cell-conditioned medium (Fig. 5), indicating that soluble TRAIL was unlikely to contribute to cytotoxicity. These findings demonstrate that NK cells expanded and activated with the K562 clone 9.mbIL21 feeder cell system utilize membrane-bound TRAIL to supplement the perforin/granzyme exocytosis pathway when eliminating neuroblastoma cells.

Effect of IFNγ on caspase-8 expression and on response to membrane-bound TRAIL on aNK cells

Since cell death in neuroblastoma cells through TRAIL-R2 is thought to be mediated chiefly through caspases-8, caspase-8 basal expression was examined by immunoblotting. In our panel of seventeen cell lines, basal expression of the p54 and p55 isoforms of caspase-8 was observed only in CHLA-79, LA-N-2, and LA-N-6 cell lines (Fig. 6A), consistent with previous reports of minimal or no caspase-8 expression in most neuroblastoma cell lines. It has been reported that IFNγ can increase caspase-8 levels and sensitize neuroblastoma cells to exogenous, soluble TRAIL. To determine whether IFNγ can sensitize neuroblastoma cells to membrane-bound TRAIL on K562 clone 9.mbIL21-expanded NK cells, we examined the effect of INF-γ on caspase-8 expression in four cell lines exhibiting no detectable basal expression. After a 24-hour pretreatment of neuroblastoma cells with exogenous IFNγ, caspase-8 expression was up-regulated strongly in SMS-KCNR and CHLA-42 cells, moderately in LA-N-1, with no expression detected in CHLA-51 cells (Fig. 6B). In functional experiments, pretreatment with IFNγ for 24 hours reproducibly increased the role of TRAIL in aNK cell cytotoxicity against SMS-KCNR and CHLA-42 cells, but not against LA-N-1 and CHLA-51 cells (Fig. 6C). These findings demonstrate that exogenous IFNγ can enhance the role of membrane-bound TRAIL in aNK cytotoxicity against some, though not necessarily all, neuroblastoma cells.

Activated NK cells are known to endogenously release IFNγ. Endogenous IFNγ is detectable in the conditioned medium of NK cells expanded and activated with K562 clone 9.mbIL21 feeder cells, and is increased six-fold to approximately 1,300 pg/ml and 400 pg/ml in the conditioned medium of those expanded aNK cells derived from healthy and neuroblastoma patient donors, respectively, in response to co-culture with neuroblastoma cells and the clinical anti-GD2 antibody ch14.18 (manuscript submitted). To explore whether endogenously produced IFNγ might exert a similar effect as did exogenous IFNγ, a neutralizing anti-IFNγ antibody was employed in the 8-hour cytotoxicity assay at a final concentration of 20 µg/ml (providing an antibody : IFNγ ratio of > 1,000). However, although a significant increase in cell survival after pretreatment of aNK cells with neutralizing anti-IFNγ antibody was observed in SMS-KCNR cells (p < 0.001), no effect was observed in CHLA-42 cells (p = 0.19, Fig. 6D). Combination of anti-IFNγ neutralizing antibody with anti-TRAIL neutralizing antibody had no significant effect on cell survival.
beyond that of anti-TRAIL antibody alone (p = 0.74 for SMS-KCNR, p = 0.71 for CHLA-42). Although an *in vitro* model, these results suggest that IFNγ produced by aNK cells may increase the sensitivity of some neuroblastoma cell lines to membrane-bound TRAIL, while leaving open the possibility of involvement of additional levels of mechanistic regulation.

**DISCUSSION**

Evidence has previously been presented that recombinant soluble TRAIL can unexpectedly induce increases in neuroblastoma cell numbers.\(^4,5\) This proliferative effect of soluble TRAIL on neuroblastoma cells\(^4,5\) contrasts with the known role of membrane-bound TRAIL expressed on NK cells in contributing to cytolysis of tumor cell types other than neuroblastoma.\(^13–16\) Addressing these disparate effects of soluble versus membrane-bound TRAIL, it is reported that membrane-bound death ligands can deliver qualitatively different signals than soluble ligands.\(^3,30\) To understand the effect of NK cell expression of membrane-bound TRAIL during cytolytic attack on neuroblastoma cells, a neutralizing antibody against TRAIL was employed and found to modestly inhibit the cytolysis of fourteen of seventeen neuroblastoma cell lines (Fig. 2). This suggests that, despite down-regulation of caspase-8 by gene methylation in many neuroblastoma cell lines and clinical neuroblastomas,\(^17–19,21\) a remnant of the TRAIL-R2-mediated cell death signaling pathway persists or can be induced in most neuroblastoma cells upon interaction with aNK cells. Thus, although recent reports indicate that neuroblastoma cells fail to undergo apoptosis\(^17–20\) or proliferate\(^4,5\) in response to anti-TRAIL agonistic antibodies or soluble TRAIL, those findings cannot be extrapolated to suggest that membrane-bound TRAIL expressed on aNK cells is ineffectual or growth-promoting. The findings presented here indicate that, rather than delivering a proliferative signal to neuroblastoma cells through TRAIL-R2, aNK cells utilize membrane-bound TRAIL to supplement the perforin/granzyme cytotoxic pathway, contributing to target cell lysis. We have recently observed that the tumors of approximately two-thirds of high-risk neuroblastoma patients at diagnosis express TRAIL-R2 mRNA at medium or high levels (manuscript in preparation), which is consistent with the possibility that TRAIL-R2 might participate in aNK cell-mediated therapies of many neuroblastomas. Notably, the role of TRAIL-R2 in aNK cytotoxicity against neuroblastoma cells *in vivo* might potentially be larger than determined here *in vitro*, since human neuroblastoma cell lines expressing no detectable caspase-8 *in vitro* can reportedly express high levels when grown as xenografts in immunocompromised mice.\(^32\)

At least three mechanisms potentially account for the supplemental effect of membrane-bound TRAIL. These include caspase-8 up-regulation by IFNγ produced at or near the immunological synapse; caspase-independent cell death; and clustering of membrane-bound TRAIL molecules. Regarding the first of these mechanisms, exogenous IFNγ can induce caspase-8 expression in neuroblastoma cell lines and can thereby sensitize them to soluble TRAIL and/or agonistic anti-TRAIL-R2 antibody.\(^20,31\) as we also show for membrane-bound TRAIL in Fig. 6B and 6C. However, the findings presented here suggest that induction of caspase-8 expression by IFNγ produced endogenously from K562 clone 9.mbIL21-activated NK cells might be sufficient to only partially sensitize a subset of neuroblastoma cell lines to membrane-bound TRAIL *in vitro* (Fig. 6D). IFNγ is known to

\(^4\) Sheard et al. Page 10

\(^5\) J Immunother. Author manuscript; available in PMC 2014 June 20.
induce up-regulation of MHC class I molecules, thereby sensitizing target cells to cytotoxic T lymphocytes and desensitizing to NK cells, adding complexity to the impact of IFNγ production by NK cells.

Regarding the second possible mechanism, caspase-independent cell death downstream of death-receptor ligation has been reported previously, and may involve receptor-interacting protein RIP and NF-κB. The third potential mechanism involves the degree of clustering of TRAIL as trimers that subsequently trimerize TRAIL-receptors. It has been reported that spatial fixation of adjacent TRAIL trimers results in secondary multimerizations into supramolecular clusters leading to increases in TRAIL-receptor-mediated cell death. The degree of multimerization of TRAIL trimers on aNK cell surfaces may be affected by localization of lipid rafts, cytoskeletal support, or ligand auto-aggregation. The multimerization of TRAIL-receptor trimers on target cells would send a qualitatively different signal than single trimers of soluble TRAIL. The relative contributions of endogenous IFNγ, caspase-independent cell death, and the multimerization of TRAIL-receptor trimers to aNK cell cytolysis of neuroblastoma cells is currently unresolved.

NK cells are thought to contribute to the elimination of neuroblastoma cells during treatment of patients with anti-GD2 antibody ch14.18. A phase I clinical trial is in preparation to evaluate combined administration of ch14.18 and autologous NK cells expanded and activated using the K562 clone 9.mbIL21 system. Our findings show that despite the potential growth-promoting effect of soluble TRAIL, membrane-bound TRAIL on aNK cells derived from healthy donors or neuroblastoma patients can supplement the perforin/granzyme pathway in aNK cell-mediated cytotoxicity against most neuroblastoma cell lines. Altogether, these findings support a role for TRAIL-R2 as a receptor for aNK cell-mediated cytotoxicity against neuroblastoma cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Cancer Institute (2RO1 CA60104, 5P01 CA81403), the ThinkCure Foundation, the Bogart Pediatric Cancer Research Program, the T.J. Martell Foundation, the Al Sherman Foundation, the Anna Bing Arnold endowment, and an Immunology Research Grant from BD Biosciences.

REFERENCES


29. StataCorp. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP; 2009.


Figure 1.
Expression of TRAIL-R2 by neuroblastoma cell lines. NK cells were enriched from healthy donor PBMC by removing other cell populations by magnetic cell sorting (negative selection) and then activated for three weeks with IL-2 (40 ng/ml) plus IL-15 (10 ng/ml). A) Inverse association between TRAIL-R2 mRNA expression and percent tumor cell survival measured in an aNK cytotoxicity assay. Cell line expression of TRAIL-R2 mRNA was determined by microarray gene expression and is plotted as fluorescent units (F.U., represented as bars, with units shown on the left Y-axis) in relationship to % Tumor Cell Survival measured as relative percentage of calcein-AM fluorescence after an 8-hour co-incubation with aNK cells (solid line, right Y-axis) (averages based on at least 5 independent wells per condition). Spearman correlation coefficient = −0.60 (p = 0.023). The order of appearance of cell lines was chosen according to decreasing sensitivity to aNK cells. B) Surface TRAIL-R2 protein expression, as determined by flow cytometry in relationship to % Tumor Cell Survival after an 8-hour co-incubation with aNK cells. Values for the ratio of mean fluorescent intensity (MFI Index) were calculated as (MFI of experimental) / (MFI of isotype control). Means ± s.d. from three independent experiments are shown. Spearman correlation coefficient = −0.55 (p = 0.022). In A and B, standard deviations for values of % Tumor Cell Survival are not shown for reason of clarity, but did not exceed 10% for any cell line except SMS-KAN cells (16%). C) Positive correlation between expression of TRAIL-R2 mRNA and corresponding surface protein. D) Surface protein expression of TRAIL-R1.
HeLa cells were included as a positive control. The solid line denotes the staining level that is equivalent to that of the isotype-matched irrelevant antibody control.
Figure 2.
Effects of membrane-bound and soluble TRAIL on neuroblastoma cell lines. A) Effect of membrane-bound TRAIL in the cytotoxicity of aNK cells against neuroblastoma cells. aNK cells were expanded and activated with IL-2 and IL-15, as described in Materials and Methods. Calcein-AM-loaded neuroblastoma cell lines were co-incubated for 8 hours with aNK cells pre-treated with anti-CD95L (αCD95L), anti-TRAIL (αTRAIL), or anti-TNF-α (αTNF) neutralizing antibodies (50 µg/ml), or with concanamycin A (CMA) (1 µg/ml) at 37°C for 90 minutes, and measured by digital imaging microscopy. Asterisks indicate those treatment groups co-incubated with aNK cells plus anti-TRAIL neutralizing antibody that differed significantly (p < 0.05) from the corresponding group co-incubated with aNK cells alone. In some instances, neuroblastoma cell survival was significantly elevated in groups involving co-incubation with NK cells that had been pretreated with anti-TNF neutralizing antibody; however, these effects were small relative to that of the anti-TRAIL group and their significance is therefore not annotated. Asterisks were omitted for cells co-incubated with aNK cells plus CMA, since they were highly significantly different in every case. Results are representative of at least 3 individual experiments containing a minimum of 5 replicate wells per condition for each cell line. B) Effect of soluble recombinant TRAIL on neuroblastoma cell numbers. Neuroblastoma cell lines were seeded into 96-well plates and...
incubated with a wide range of concentrations of recombinant TRAIL for 96 hours. Cells were then loaded with fluorescein diacetate and cell numbers were determined using digital image microscopy. Results are representative of at least three independent experiments, each containing ten replicate wells per cell line; asterisks represent statistical significance (p < 0.05) across all experiments.
Figure 3.
A high level of TRAIL expression on aNK derived from PBMC by co-culture with lethally irradiated K562 clone 9.mbIL21 feeder cells plus IL-2. A) aNK cell surface expression of TRAIL analyzed by flow cytometry. Healthy donor PBMC were incubated for fourteen days with IL-2 alone (5 ng/ml), K562 clone 9.mbIL21 feeder cells alone (effector: target ratio = 2:1), IL-2 plus feeder cells, or neither, and compared with freshly thawed, non-cultured PBMC from the same donor. Inspection under a light microscope and on flow cytometric light scatter plots indicated that lysis of the very large, lethally irradiated K562 clone 9.mbIL21 myeloid feeder cells was complete by day 14 (data not shown). Cells were stained simultaneously with six antibodies against CD16, CD56, CD19, CD3, TRAIL, and CD14 (CD14 not shown), plus the viability dye DAPI. Blue colored dots and lines represent NK cells as identified by the gate shown on the CD56×CD16 dot-plots; all other (non-NK) cells
are colored maroon. In the histogram overlays, histograms representing TRAIL expression (solid lines) are overlaid with histograms representing isotype-matched control antibody staining (dashed lines) obtained by gating on fluorescence-minus-one controls performed for each treatment group. The data presented is from one donor and is representative of three healthy donors. B) The total number of cells in each treatment group was determined at various time-points by manual counting using a hemocytometer while excluding dead cells according to their staining with trypan blue. The starting number of PBMC was $10^7$ for each group.
Figure 4.
aNK cells expanded and activated from PBMC using K562 clone 9.mbIL21 feeder cells plus IL-2 use TRAIL to supplement perforin/granzyme-mediated cytotoxicity against neuroblastoma cells. Calcein-AM-loaded neuroblastoma cell lines were co-incubated for 12 hours with aNK cells pretreated with neutralizing antibodies (50 µg/ml) against CD95L, TRAIL, or TNF-α, or with CMA (1 µg/ml) for 90 minutes at 37°C. Percent survival was then measured by digital imaging microscopy. Asterisks mark significant differences (p < 0.05) between those treatment groups co-incubated with aNK cells plus anti-TRAIL neutralizing antibody and those from the corresponding group co-incubated with aNK cells alone. In some instances, neuroblastoma cell survival at this 12 hour time-point was significantly elevated in groups involving co-incubation with NK cells that had been pretreated with anti-CD95L and anti-TNF neutralizing antibodies; however, these effects were small relative to that of the anti-TRAIL group and their significance is therefore not annotated. Asterisks were also omitted for cells co-incubated with aNK cells plus CMA, since they were highly significantly different in every case. Each experiment contained a minimum of 6 replicate wells per condition for each cell line.
Figure 5.
Membrane-bound TRAIL but not soluble TRAIL supplements aNK cytotoxicity against neuroblastoma cells. The binding of anti-TRAIL neutralizing antibody was constrained to membrane-bound TRAIL on K562 clone 9.mbIL21-expanded aNK cells but not to soluble TRAIL in the culture medium as follows: aNK cells that were cultured with IL-2 for 72 hours were pelleted and their conditioned medium (CM) was saved; after cells were washed twice to remove residual soluble TRAIL, they were pretreated with anti-TRAIL neutralizing antibody for 90 minutes and then washed twice again to remove any unbound antibody. aNK cells with bound anti-TRAIL antibody were then cultured with calcein-AM-loaded SMS-KAN and CHLA-134 cells for 8 hours without or with their own conditioned medium (75% final concentration). The data shown are from one PBMC donor, and are representative of three healthy donors examined.
Figure 6.
Effect of IFNγ on the sensitivity of neuroblastoma cell lines to aNK cell-mediated cytotoxicity. A) Basal expression of the p54 and p55 isoforms of uncleaved caspase-8 in the panel of 17 neuroblastoma cell lines, as determined by immunoblotting. Staining for β-actin was included as a control for the accuracy of protein loading. B) Induction of caspase-8 expression by exogenous IFNγ. Four cell lines with no detectable basal expression of caspase-8 were cultured without or with IFNγ (10 ng/ml) for 24 hours and then lysed for immunoblotting. C) Functional effect of exogenous IFNγ on the sensitivity of neuroblastoma cells to membrane-bound TRAIL expressed on aNK cells. In the representative experiment shown, neuroblastoma cells were pretreated with 10, 1, or 0.1 ng/ml IFNγ for 24 hours, K562 clone 9.mbIL21-expanded aNK cells were or were not pretreated anti-TRAIL neutralizing antibody for 90 minutes, and then cells were co-incubated for 8 hours in a calcein-AM cytotoxicity assay as in Figure 2. Across the three independent experiments performed, the groups receiving 1 ng/ml or 10 ng/ml of IFNγ exhibited effects of anti-TRAIL neutralizing antibody with p values of 0.040 and 0.055, respectively. D) Effect of anti-IFNγ neutralizing antibody on the response of neuroblastoma cells to aNK cytotoxicity. K562 clone 9.mbIL21-expanded aNK cells were or were not treated with anti-TRAIL neutralizing antibody for 60 minutes, and then seeded into a 96-well plate where specified wells contained anti-IFNγ neutralizing antibody (20 µg/ml final concentration). Thirty minutes later, neuroblastoma cells were added, followed by an 8-hour aNK calcein-AM cytotoxicity assay. The results shown are representative of three independent experiments.