Clinical Cancer Research

Activated Natural Killer Cells in Combination with Anti-GD2 Antibody Dinutuximab Improve Survival of Mice after Surgical Resection of Primary Neuroblastoma

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Abstract

Purpose: Immunotherapy of neuroblastoma that remains after myeloablative chemotherapy with anti-GD2 antibody dinutuximab has increased the two-year event-free and overall survival of high-risk neuroblastoma patients; however, 40% of patients develop recurrent disease during or after this treatment. To determine the potential of such antibody-based immunotherapy earlier in treatment, a mouse model was developed in which surgical resection of the primary tumor was followed by therapy of residual disease with dinutuximab combined with *ex vivo*–activated human natural killer (aNK) cells.

Experimental Design: The effect of combining dinutuximab with human aNK cells was determined *in vitro* with cellular cytotoxicity and Matrigel invasion assays. The *in vivo* efficacy of dinutuximab and aNK cells against neuroblastoma was assessed following resection of primary tumors formed by two cell lines or a patient-derived xenograft (PDX) in immunodeficient NOD-scid gamma mice.

Results: *In vitro*, the combination of aNK cells and dinutuximab caused cytotoxicity and decreased invasiveness of three human neuroblastoma cell lines. Treatment of mice with dinutuximab combined with aNK cells after surgical resection of primary intrarenal tumors formed by two cell lines or a PDX decreased tumor cells in liver and bone marrow as evaluated by histopathology and bioluminescence imaging. Survival of mice after resection of these tumors was most significantly increased by treatment with dinutuximab combined with aNK cells compared with that of untreated mice.

Conclusions: The combination of dinutuximab and adoptively transferred human aNK cells following surgical resection of primary neuroblastomas significantly improves survival of immunodeficient mice.

Introduction

Neuroblastoma is a malignancy of neuroectodermal origin and is the most common extracranial solid tumor of childhood (1–4). Despite improvements in multimodal therapy, the 5-year event-free survival (EFS) for patients with high-risk disease remains approximately 45% (2, 3, 5–8). Nearly 80% of patients respond to induction therapy, which includes chemotherapy and surgical resection of the primary tumor (8). However, this initial therapy requires improvement as only 22% and 56% of patients achieve complete and partial clinical responses (8). Immunotherapy with anti-disialoganglioside (GD2) mAb (dinutuximab) could improve the efficacy of induction therapy since its use after myeloablative therapy, supported by autologous hematopoietic stem cell transplantation, has increased the 2-year EFS and overall survival (9). We have previously shown that cryopreserved, *ex vivo*–propagated and activated natural killer (aNK) cells maintain their ability to induce antibody-dependent cellular cytotoxicity (ADCC) when combined with dinutuximab and improve survival of immunodeficient mice with disseminated neuroblastoma (10).

The impact of immunotherapy with dinutuximab combined with adoptively transferred aNK cells on neuroblastoma cells after surgical resection of the primary tumor is yet to be examined. We developed a model in immunodeficient NOD-scid gamma (NSG) mice that simulates a clinical scenario in which immunotherapy is given following surgical resection. The primary tumor is established by injecting human neuroblastoma cells into the kidney of NSG mice, and it is resected after growing for 7 days. Although resection is grossly complete, untreated mice have tumor cells that are detectible by bioluminescence imaging at the primary site 1 day after resection and in liver and bone marrow by imaging and histopathology within 3 to 4 weeks (11). We show that dinutuximab combined with adoptively transferred aNK cells following surgical resection decreases neuroblastoma growth in liver and bone marrow and increases survival of mice.

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Translational Relevance

Children with high-risk neuroblastoma often achieve clinically defined remission during or following induction phase chemotherapy and surgery, consolidation phase myeloablative chemotherapy, and postconsolidation phase antibody immunotherapy. Overall, 45% become long-term survivors, but the remainder develop refractory or recurrent disease, and nearly all subsequently succumb. Immunotherapy with the anti-GD2 antibody dinutuximab following myeloablative chemotherapy improves 2-year event-free and overall survival. Dinutuximab, when combined with chemotherapy, also can be effective against persistent or recurrent disease. Experimentally, dinutuximab with *ex vivo*-activated natural killer (aNK) cells causes antibody-dependent cellular cytotoxicity *in vitro* and tumor responses in mice. This combination has not been examined experimentally or clinically for efficacy after surgical removal of the primary tumor. Our preclinical study demonstrates that treatment with the combination of dinutuximab and aNK cells after surgical resection of bulk disease significantly inhibits residual tumor growth and improves survival. This strategy could further improve survival of children with high-risk neuroblastoma.

Materials and Methods

Neuroblastoma cell lines and patient-derived xenograft

CHLA-136, CHLA-255, and SH-SY5Y human neuroblastoma cell lines as well as neuroblastoma patient-derived xenograft (PDX) COG-N-415x cells were derived from patients with progressive disease (12–17). CHLA-136 and CHLA-255 cells were obtained from the Children's Oncology Group (COG) Cell Culture and Xenograft Repository (www.COGcell.org). SH-SY5Y cells were obtained from ATCC. CHLA-136 cells have a high level of GD2 expression (Supplementary Fig. S1) and have genomic amplification of *MYCN* (14). CHLA-255 cells have a high level of GD2 expression and express c-MYC protein, thereby representing high-risk, undifferentiated/poorly differentiated neuroblastoma lacking *MYCN* proto-oncogene amplification (18–20). Notably, patients expressing MYCN or c-MYC protein detected by immunofluorescence have similar and significantly low survival (20). SH-SY5Y cells have a medium level of GD2 expression and are *MYCN*-nonamplified (21). COG-N-415x PDX cells have a medium to high level of GD2 expression and have amplification of *MYCN* and mutation of *ALK* (F1174L; provided by Dr. C. Patrick Reynolds, www.COGcell.org). These three cell lines and PDXs represent the heterogeneity of high-risk human neuroblastomas. The firefly luciferase (Fluc) gene was transduced into SH-SY5Y (SH-SY5Y-Fluc), CHLA-136 (CHLA-136-Fluc) cells, and CHLA-255 (CHLA-255-Fluc) cells using a lentiviral vector, as described previously (10, 17).

aNK cells, reagents, and cell culture

aNK cells from healthy human donors were propagated and activated *ex vivo* by incubating peripheral blood mononuclear cells (PBMC) in RPMI1640 medium and 10% heat-inactivated FBS (Omega Scientific; catalog no. FB-02) containing human IL2 (10 ng/mL, 100 μ/mL; PeproTech; catalog no. 200-02) and irradiated (100 Gy) K562-mbIL21 feeder cells genetically engineered to express immunostimulatory molecules including CD137 ligand and membrane-bound IL21, as described previously (10, 17, 22). At day 14, aNK cells were cryopreserved in aliquots. Upon thawing, aNK cells were either allowed to recover for *in vitro* assays by culturing in RPMI1640 medium and 10% FBS with IL2 for 2 days or were thawed and immediately injected intravenously into mice.

The human neuroblastoma cell line SH-SY5Y-Fluc was maintained in RPMI1640 (Corning; catalog no. 10-040-CV). Human neuroblastoma cell lines CHLA-136-Fluc and CHLA-255-Fluc were maintained in Iscove's Modified Dulbecco's medium (University of Southern California Stem Cell Core, Los Angeles, CA). All media included 10% FBS and 2 mmol/L L-glutamine (Gibco by Life Technologies; catalog no. 25030-081). Cell lines were maintained at 37°C in 5% CO_2 until 80% confluence was reached, and then they were harvested using 0.5% trypsin-EDTA (Corning; catalog no. 25-052-CL). All cell lines were screened routinely for *Mycoplasma*, and donor cell identity was authenticated by short tandem repeat multiplex assay using the AmpFLSTR Identifiler PCR Amplification Kit (Applied Biosystems; catalog no. 4322288).

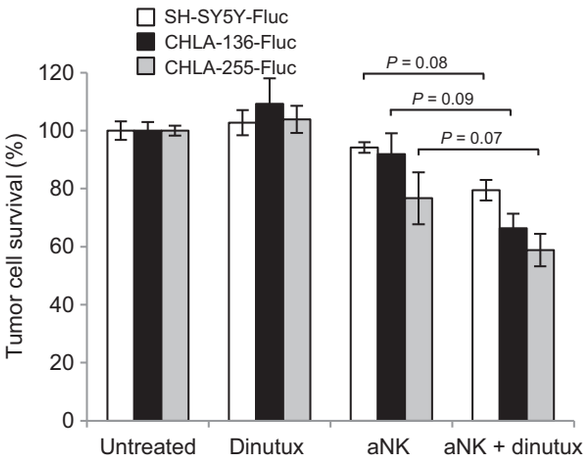
The following reagents were used: dinutuximab (United Therapeutics), recombinant human IL2 (PeproTech; catalog no. 200-02), and recombinant human IL15 (PeproTech; catalog no. 200-15).

Flow cytometry

Surface staining for GD2 was performed on SH-SY5Y-Fluc, CHLA-136-Fluc, CHLA-255-Fluc, and COG-N-415x cells. Briefly, cells were washed twice in FACS buffer (PBS with 5% BSA; Thermo Fisher Scientific SH3057402) and centrifuged for 8 minutes at 100 × *g*. Fc-receptors were blocked by incubation in human Fc-blocker for 10 minutes at 4°C (Human True Stain FcX, BioLegend 422302), followed by incubation with anti-human GD2 antibody (BioLegend 357306) and isotype-matched irrelevant control (BD Pharmingen 340754) for 1 hour in the dark at 4°C. Cells were then washed twice in FACS buffer, and DAPI was added (0.5 ng/mL final concentration) to each tube. Flow cytometry was conducted using a LSR II flow cytometer (BD Biosciences), and BD FACSDiva software was used to collect and analyze data. Experiments were repeated a minimum of three times. Ratios for median fluorescence intensity (MFI) index were calculated as follows: (MFI of viable cells stained with specific antibody/MFI of viable cells stained with isotype-matched irrelevant antibody).

Cytotoxicity assay

Frozen K562.mbIL21-expanded aNK cells were cultured in 10% FBS containing RPMI1640 medium with 100 μ/mL of IL2 for 48 hours. For the cytotoxicity assay, basal media containing 2% FBS was used. Neuroblastoma cell lines SH-SY5Y-Fluc, CHLA-136-Fluc, and CHLA-255-Fluc were washed, resuspended in cell culture medium, and incubated with Invitrogen's calcein-AM (Thermo Fisher Scientific; catalog no. C1429) for 30 minutes at 37°C in 5% CO_2. The fluorescence of the calcien-AM seen by microscopy is directly proportional to the number of viable neuroblastoma cells (23). Neuroblastoma cells were washed, and 3×10^4 cells were seeded per well in a 96-well plate. After 5 minutes, 1.5×10^4 aNK cells, treated with IL2 as described above, were added to cultures with neuroblastoma cells at an effector: target (E:T) ratio of 1:2. To wells containing aNK cells, 100 μ/mL of IL2 was added. Dinutuximab was added at a concentration of



***P* values of treated versus untreated**

	Dinutux	aNK	aNK + dinutux
SH-SY5Y-Fluc	0.91	0.56	0.03
CHLA-136-Fluc	0.78	0.74	0.02
CHLA-255-Fluc	0.96	0.18	0.01

Figure 1.

Effect of anti-GD2 antibody dinutuximab (dinutux) and aNK cells on neuroblastoma cells *in vitro*. Calcein AM–based digital imaging microscopy assay showing the viability of human neuroblastoma cell lines SH-SY5Y-Fluc, CHLA-255-Fluc, and CHLA-136-Fluc following treatment with dinutuximab alone (100 ng/mL), aNK cells alone, or their combination for 6 hours. For each cell line, 3×10^4 neuroblastoma cells were seeded per well of a 96-well plate, along with 1.5×10^4 aNK cells as described in Materials and Methods (E:T ratio 1:2). Each condition contained six replicates and each experiment was performed a minimum of two times. Comparisons were made between groups using ANOVA and *post hoc* Tukey HSD tests.

100 ng/mL. The plate was incubated at 37° C in 5% CO_2 for 6 hours and survival of neuroblastoma cells was assessed using a digital imaging microscopy system, (DimScan, available from BioImaging Solutions, Inc.). Relative tumor cell survival was then calculated: (fluorescence of treated, Calcein-AM–labeled cells/ fluorescence of untreated, calcein-AM–labeled cells). aNK cells were not labeled and therefore did not contribute to the fluorescent signal. Each condition contained six replicates and each experiment was performed a minimum of two times.

Matrigel invasion assay

Frozen K562.mbIL21–expanded human aNK cells were cultured in 10% FBS-RPMI1640 with 100 μ/mL of IL2 for 48 hours. For this assay, neuroblastoma cell lines SH-SY5Y-Fluc, CHLA-136-Fluc, and CHLA-255-Fluc were pretreated using basal media containing 1% FBS in T-25 flasks for 6 hours with (i) 100 ng/mL dinutuximab, (ii) aNK cells plus 100 μ/mL of IL2, or (iii) 100 ng/mL dinutuximab and aNK cells with 100 μ/mL IL2. Following treatment, flasks were washed twice with warm PBS (Corning; catalog no. 21-031-CV) and allowed to recover for 16 hours in basal media containing 1% FBS. Biocoat 24-well Matrigel Invasion Chambers (Corning; catalog no. 354480) were allowed to equilibrate at room temperature for 10 minutes, followed by rehydration at 37°C for 2 hours. Viable cells were counted using Trypan blue exclusion, and 1×10^5 neuroblastoma cells were seeded in the top well of the chamber and incubated for 72 hours in 5% CO_2 at 37°C. Migrated cells were visualized using Fisher HealthCare PROTOCOL Hema 3 Manual Staining System and Stat Pack (Thermo Fisher Scientific; catalog no. 22-123869). Images were captured using a Zeiss Axioplan microscope and the Spot Advanced software package (version 4.0.9, Spot Imaging, Inc.). Cells that migrated to the bottom chamber were quantified using Image J software (version 1.50i, NIH, Bethseda, MD). Independent experiments were performed a minimum of three times for each cell line.

Surgical resection of human neuroblastoma and treatment of NSG mice

Six- to 8-week-old NSG mice, which lack NK, B, and T cells, were bred in-house and genotyped for colony maintenance. These mice were used for all *in vivo* experiments and cared for under pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee of Children's Hospital Los Angeles (IACUC #363-17). Neuroblastoma cell viability before injection was assessed using Trypan blue staining, and cells were washed and suspended in sterile PBS (Corning; catalog no. 21-031-CV) at a concentration of 1×10^6 neuroblastoma cells per 0.1 mL. 1×10^6 CHLA-136-Fluc, CHLA-255-Fluc, or COG-N-415x cells, which were >90% viable, were injected into the left kidney of NSG mice. After 7 days, complete surgical resection of the xenografted kidney was performed.

After surgery, mice were randomized into four treatment groups: (i) control, (ii) dinutuximab alone, (iii) aNK cells alone, or (iv) the combination of aNK cells and dinutuximab. All mice that received dinutuximab were administered 15 μg ($\sim$ 2.14 mg/m^2) by intravenous tail vein injection twice a week for 4 weeks for a cumulative dose of 17.1 mg/m^2, starting 2 days following surgical resection. This cumulative dose approximates the daily dose administered to patients (17.5 mg/m^2, range 10–25 mg/m^2) and is approximately one-fourth the cumulative monthly dose given to patients. Mice received aNK cells (1×10^7) by tail vein injection twice a week for 4 weeks starting 2 days following surgical resection. This dose is in the range of doses being used in ongoing clinical trials NCT03420963, NCT02763475, and NCT02573896. Mice receiving aNK cells were given IL2 (2 μg/mouse; 2×10^4 μ/mouse) and IL15 (5 μg/mouse; 1×10^5 μ/mouse) via intraperitoneal injection at the same time as injection of aNK cells.

Assessment of residual neuroblastoma by bioluminescence imaging and histopathology

Bioluminescence imaging (Xenogen IVIS Lumina XR System, Caliper Life Sciences) was used to quantify the overall burden of disease weekly beginning 2 days after surgical resection of the left kidney, which was performed 7 days after tumor cell injection. Total flux (photons/second) of the entire mouse after injection of CHLA-136-Fluc or CHLA-255-Fluc cells was quantified using Living Image software (Living Image 4.3.1, Caliper Life Sciences) and compared with pretreatment baseline. Incidence of distant residual tumors was determined by the presence of bioluminescence within the femurs or liver and confirmed by histopathology. Mice were euthanized when IACUC criteria for euthanasia were met, which included massive tumor burden from recurrent disease, poor eating, grooming, paralysis, and/or lethargy.

At the time of sacrifice, liver tissue and femurs from all mice were formalin-fixed and stored in 75% ethanol at 4°C. Specimens underwent serial dehydration in ethanol followed by immersion

in xylene and paraffin embedding. All tissues were cut into 5-μm sections and stained with hematoxylin and eosin (H&E) by the Department of Pathology at Children's Hospital Los Angeles (Los Angeles, CA). Two sections of liver and a single longitudinal section of both femurs were evaluated from each mouse. The presence of morphologically identifiable neuroblastoma cells in these sections was determined in a blinded manner by Dr. L. Wang, attending pathologist, who specializes in neuroblastoma.

Statistical analyses

Data for the *in vitro* cytotoxicity and Matrigel invasion assays were compared using an ANOVA and Tukey HSD *post hoc* test. The presence of neuroblastoma in liver and bone marrow, assessed as described above, was analyzed using Fisher exact test. Weekly bioluminescent flux measurements were adjusted to baseline observations and the area under the curve (AUC) was calculated. The AUC was $\log_{10}$ transformed to normalize the data, and linear regression was used to assess the treatment effects on residual disease growth ($\log_{AUC}$). Survival time is defined as the length of time between the day of tumor cell injection and the time the mice were found dead or sacrificed because of residual disease burden. Because of the large variability in survival time between groups, linear regression was performed on the $\log_{10}$ of survival time. Analyses were performed using Statistical Package for the Social Sciences version 17.0 (SPSS Inc.) and Stata 11 (StataCorp LP.). The *P* values refer to two-sided tests and a P value < 0.05 was considered significant.

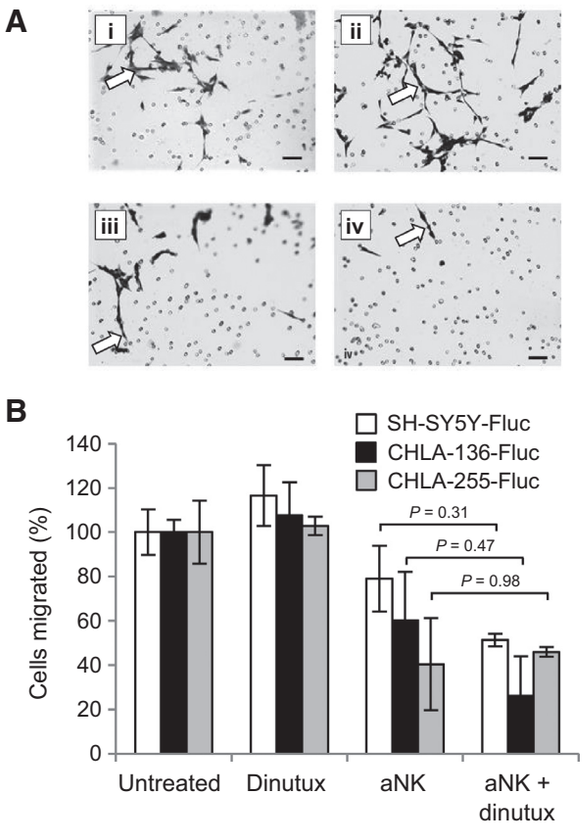
Results

Dinutuximab induces antibody-dependent cell-mediated cytotoxicity when combined with aNK cells *in vitro*

GD2 expression levels for all cell lines were assessed by flow cytometry (Supplementary Fig. S1). Each of the three cell lines used for the *in vitro* assays had greater than 97.9% of cells with GD2 expression. To assess cytotoxicity *in vitro*, neuroblastoma cell lines SH-SY5Y, CHLA-136-Fluc, and CHLA-255-Fluc were not treated (control) or treated with dinutuximab alone, aNK cells alone, or the combination of dinutuximab and aNK cells. For all three cell lines, neither dinutuximab nor aNK cells alone at a low effector:target (E:T) cell ratio of 1:2 decreased survival compared with control. The difference between aNK cells combined with dinutuximab and aNK cells alone suggested a trend but did not reach statistical significance. However, the combination of dinutuximab and aNK cells (1:2 E:T ratio) induced a significant decrease in cell viability in all three cell lines compared with control (P < 0.03 for each cell line; Fig. 1). These findings demonstrate cytotoxic effect of aNK cells on neuroblastoma cells via ADCC when combined with dinutuximab.

Dinutuximab combined with aNK cells inhibits invasion of neuroblastoma cells *in vitro*

An important hallmark of malignancy is the ability to invade the extracellular matrix to migrate to distant sites. To test whether aNK cells and/or dinutuximab affect invasiveness *in vitro*, a Matrigel assay was performed using SH-SY5Y-Fluc, CHLA-136-Fluc, and CHLA-255-Fluc neuroblastoma cells and control medium, dinutuximab alone, aNK cells alone, or the combination of dinutuximab and aNK cells. When neuroblastoma cells were treated with either aNK cells alone or dinutuximab alone, we observed no significant difference in subsequent cellular migration compared with control untreated cells (Fig. 2A and B). There was no significant difference in invasion when dinutuximab was combined with aNK cells compared with aNK cells alone. However, the combination of aNK cells and dinutuximab significantly decreased subsequent neuroblastoma cell invasion of all three cell lines into Matrigel compared



***P* values of treated versus untreated**

	Dinutux	aNK	aNK + dinutux
SH-SY5Y-Fluc	0.691	0.528	0.045
CHLA-136-Fluc	0.986	0.354	0.045
CHLA-255-Fluc	0.997	0.018	0.029

Figure 2.

Effect of activated NK cells and dinutuximab on neuroblastoma cell invasion *in vitro*. Neuroblastoma cell lines SH-SY5Y-Fluc, CHLA-255-Fluc, and CHLA-136-Fluc were treated with vehicle control, dinutuximab alone (100 ng/mL), aNK cells alone (E:T ratio 1:1 maintained with 100 μ/mL of IL2), or dinutuximab and aNK in combination. 1×10^5 neuroblastoma cells from each treatment group were seeded into the top chamber of a Biocoat 24-well Matrigel Invasion Chamber (Corning), incubated for 72 hours, and then cells that migrated to the bottom chamber were quantified. **A,** Photomicrographs of SH-SY5Y-Fluc cells which traversed the Matrigel interface, from the following treatment groups: (i) untreated control, (ii) dinutuximab, (iii) aNK cells, and (iv) aNK cells + dinutuximab. Neuroblastoma cells appear dark and elongated (arrows), whereas the small round circles are the microscopic pores in the Matrigel. Scale bar, 100 μm. **B,** Percent of migrated neuroblastoma cells following treatment with dinutuximab alone, aNK cells alone, or combination treatment. Comparisons were made between groups using ANOVA and *post hoc* Tukey HSD tests.

with control medium (SH-SY5Y-Fluc, $P = 0.045$; CHLA-136-Fluc, $P = 0.045$; CHLA-255-Fluc, $P = 0.029$).

Dinutuximab combined with aNK cells inhibits growth of residual neuroblastoma following surgical resection of the primary tumor

For the *in vivo* studies, we utilized a tumor resection model of neuroblastoma, whereby a xenografted kidney was surgically removed 1 week following tumor cell injection. Our surgical model demonstrates a significant reduction of neuroblastoma disease burden when comparing pre- and postresection bioluminescent imaging (Fig. 3A). A 95% reduction in bioluminescent flux (photons/sec) is observed following primary tumor removal ($P < 0.001$; Fig. 3B).

The ability of dinutuximab and adoptively transferred aNK cells to inhibit establishment of detectable disease in liver and bone marrow after surgical resection of the kidney with primary neuroblastoma was determined using CHLA-136-Fluc, CHLA-255-Fluc, and PDX COG-N-415x cells (15). Following resection at day 7, mice were randomized to control, dinutuximab alone, aNK cells alone, and aNK cells plus dinutuximab treatment groups. The presence of neuroblastoma in liver or bone marrow was analyzed by bioluminescence weekly for CHLA-255-Fluc and CHLA-136-Fluc cell lines and by histologic evaluation at sacrifice for the PDX model.

Bioluminescent imaging of mice injected with CHLA-136-Fluc cells revealed disease in the liver of nearly all but only rarely in the femurs of untreated control mice (Fig. 4A and B). Treatment with aNK cells alone or in combination with dinutuximab after resection of CHLA-136-Fluc primary tumor caused a significant decrease in the incidence of bioluminescence within the liver when compared with control mice ($P < 0.01$ for both; Fig. 4A). The incidence of bioluminescent signal within liver did not significantly decrease when aNK cells and dinutuximab were compared with aNK cells alone. The infrequency of bioluminescence in femurs precluded identifying an effect of treatment on disease in femurs.

In mice xenografted with CHLA-255-Fluc cells, combination therapy after resection significantly decreased the incidence of bioluminescence in both livers and femurs ($P < 0.05$; Fig. 4A and B). In contrast, monotherapy with dinutuximab or aNK cells did not decrease bioluminescence in these sites. A statistical difference in the incidence of bioluminescent signal within liver and femur was not observed between groups treated with aNK cells and dinutuximab versus aNK cells alone.

For mice xenografted with COG-N-415x cells, liver and bone marrow were evaluated by histology for the presence of neuroblastoma following sacrifice. Similar to findings for CHLA-255-Fluc, there was a significant reduction in the incidence of neuroblastoma present in liver and bone marrow by combination treatment with dinutuximab and aNK cells ($P < 0.05$; Fig. 4E and F). Monotherapy with dinutuximab or aNK cells did not significantly decrease detectable neuroblastoma in liver or bone marrow.

In addition to evaluating the incidence of distant tumor growth, we also assessed the overall burden or quantity of residual disease in mice xenografted with CHLA-136-Fluc and CHLA-255-Fluc cells. We measured the burden of residual disease by measuring total luminescence from each mouse weekly (Fig. 5A). In mice xenografted with CHLA-136-Fluc and CHLA-255-Fluc cells, we observed a significant decrease in residual disease burden after treatment with aNK cells alone or with the combination of aNK cells and dinutuximab as compared with control (Fig. 5B). No significant difference was observed when comparing mice treated with aNK cells alone compared with combination therapy. Together, these data demonstrate the positive effect of aNK cells alone and in combination with dinutuximab in reducing the incidence and burden of distant disease following surgical resection of the primary tumor.

Dinutuximab combined with aNK cells increases survival following surgical resection of the primary tumor

In the resection model of neuroblastoma, survival of mice xenografted with CHLA-136-Fluc cells was significantly increased following aNK cells alone with a 16-day increase in mean survival ($P < 0.001$). The combination of aNK cells and dinutuximab further increased survival compared with both control (mean survival 83.1 vs. 51.6 days) and aNK cells alone (mean survival 83.1 vs. 71.2; $P < 0.05$ for both; Fig. 6). Survival of mice xenografted with CHLA-255-Fluc cells was not increased in the group

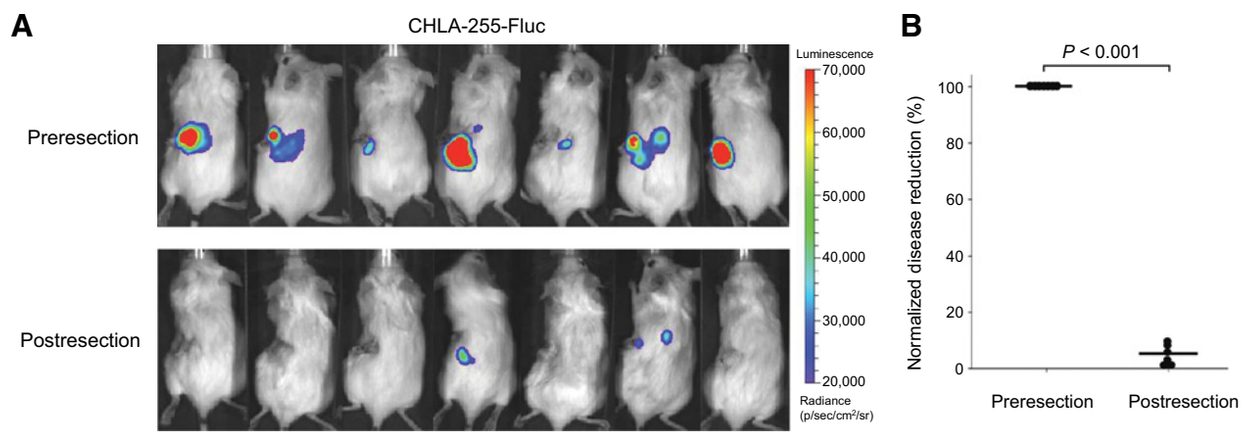


Figure 3.

Disease reduction following surgical resection of primary neuroblastoma. Mice underwent implantation of 1×10^6 CHLA-255-Fluc neuroblastoma cells followed by surgical removal of the primary tumor one week later. **A,** Bioluminescent images taken 7 days post neuroblastoma cell injection and just prior to tumor removal. Postresection images of mice were taken one day after resection and compared with preresection baseline. **B,** Surgical resection of the primary tumor resulted in a 95% reduction in disease burden.

receiving aNK cells alone compared with control mice, but was significantly increased in mice treated with the combination of dinutuximab and aNK cells compared with control (mean survival increased by 13.8 days; $P = 0.015$). In mice xenografted with COG-N-415x cells, monotherapy with dinutuximab increased mean survival by 4.8 days and aNK cells increased mean survival by 5.3 days as compared with control ($P < 0.05$ for both; Fig. 6). Moreover, survival of mice xenografted with COG-N-415x cells was greatest in the group receiving the combination of dinutuximab with aNK cells, with a 10.4-day increase in mean survival compared with control ($P < 0.001$). Dinutuximab combined with aNK cells significantly increased survival over aNK cells alone following the resection of primary tumors created by both CHLA-136-Fluc and COG-N-415x cells. These data demonstrate that following resection of the primary neuroblastoma, treatment with aNK cells alone increased survival of mice with two of three xenograft models but that the combination dinutuximab and aNK cells was most effective, prolonging survival of mice with all three xenograft models.

Discussion

We hypothesized that disease control after surgical resection of the primary tumor in patients with high-risk neuroblastoma can be improved by treatment with adoptively transferred aNK cells and anti-GD2 dinutuximab. To test this hypothesis, we developed a model of high-risk neuroblastoma using immunodeficient NSG mice. Following surgical resection of the primary tumor formed in the kidney by human neuroblastoma cell lines CHLA-136-Fluc or CHLA-255-Fluc or PDX COG-N-415x, we show that survival of mice is most effectively increased by adoptive transfer of human aNK cells with dinutuximab. These data support consideration of therapy with adoptively transferred aNK cells and dinutuximab after surgical disease reduction.

Our model generates primary human neuroblastomas in the kidney of NSG mice that can be surgically removed, which increases survival (11). Although we show that surgery removes 95% of disease, residual neuroblastoma cells can be detected consistently with bioluminescent imaging and histopathology in liver and bone marrow, which are sites of disease in patients. We demonstrate that treatment with aNK cells combined with dinutuximab after resection decreases the incidence and quantity of detectable neuroblastoma within liver and bone marrow and, importantly, increases survival of mice.

Several mechanisms could contribute to the eradication of residual neuroblastoma cells in primary and distant sites after surgical resection. First, circulating neuroblastoma cells could be decreased by direct cytotoxicity of aNK cells alone or by ADCC mediated by dinutuximab and aNK cells leaving fewer available to

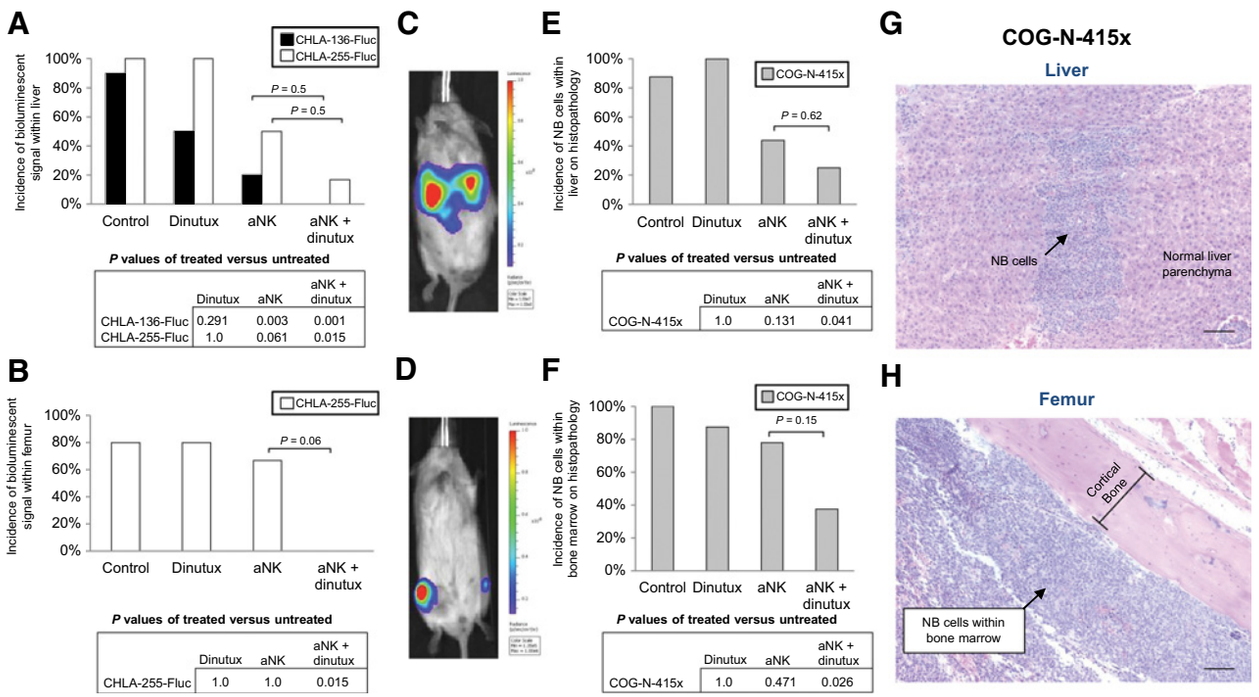
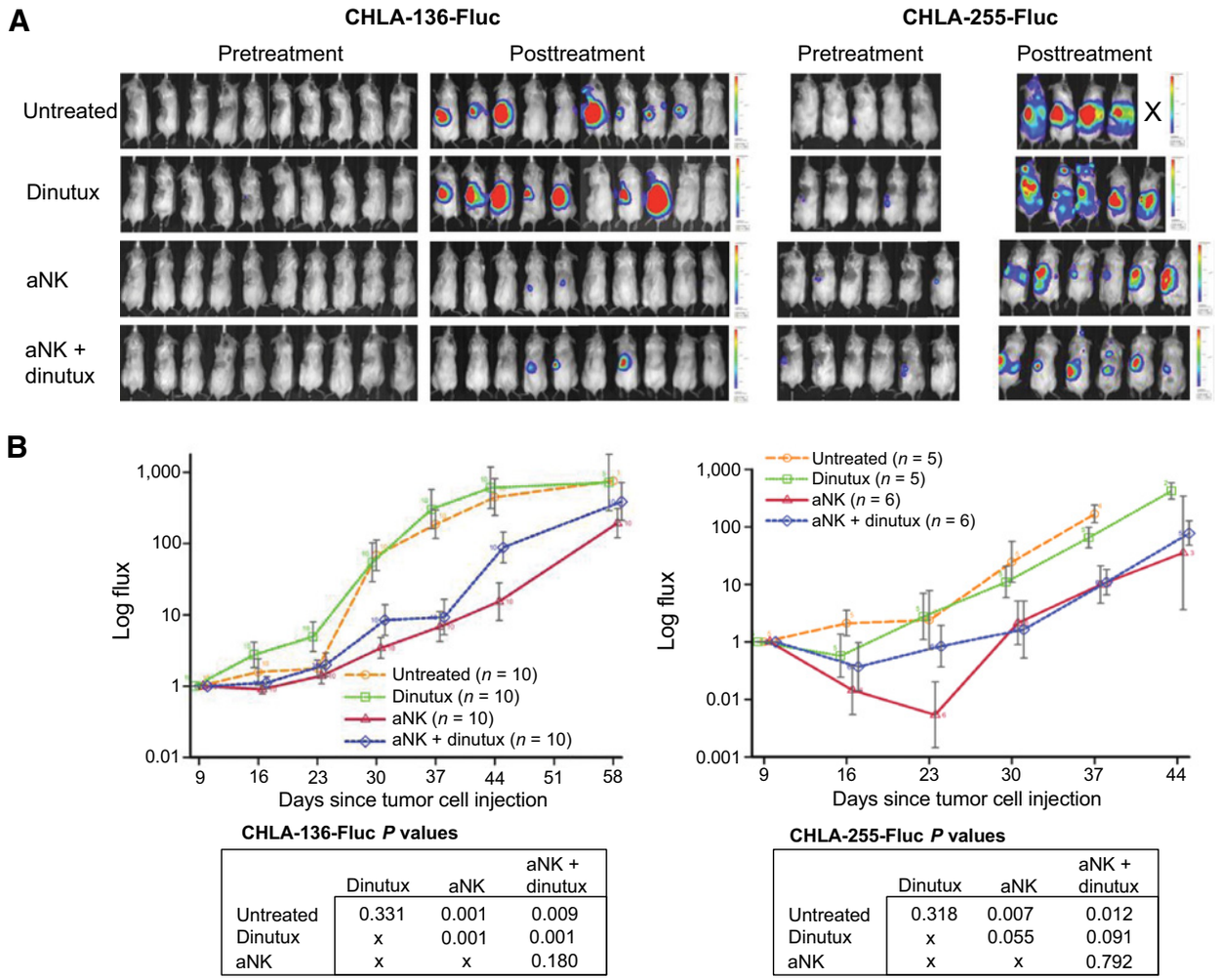


Figure 4.

Effect of activated NK cells combined with dinutuximab on residual neuroblastoma *in vivo*. **A,** Surgical resection model of neuroblastoma was utilized, in which mice were injected with 1×10^6 neuroblastoma cells (CHLA-136-Fluc, $n = 40$; CHLA-255-Fluc, $n = 22$; COG-N-415x, $n = 33$) into the left kidney, followed by complete resection of the xenografted kidney one week later. Mice were then randomized into 4 treatment groups: (i) control, (ii) dinutuximab (15 μg/mouse) (iii) 1.0×10^8 aNK cells, or (iv) 1.0×10^8 aNK cells and dinutuximab. Mice receiving aNK cells were given IL2 (2 μg/mouse) and IL15 (5 μg/mouse) via intraperitoneal injection. All treatments were administered twice a week for a total of 4 weeks. **A** and **B,** Incidence of residual neuroblastoma tumor growth following resection of CHLA-136-Fluc and CHLA-255-Fluc xenografts as determined by the presence of bioluminescent signals in the liver or femurs. CHLA-135-Fluc cells were rarely found within the bone marrow and therefore not shown. **C,** Bioluminescent imaging of residual disease within the liver of an untreated mouse following resection of CHLA-136-Fluc xenograft. **D,** Signal from the femurs of a mouse treated with dinutuximab alone following resection of CHLA-255-Fluc xenograft. **E** and **F,** Incidence of residual tumor growth following resection of COG-N-415× xenograft as determined by histopathology at the time of necropsy. **G** and **H,** H&E staining demonstrating neuroblastoma cells (arrow) within the liver and bone marrow following resection of COG-N-415x xenograft. Scale bar, 200 μm.



CHLA-136-Fluc *P* values

	Dinutux	aNK	aNK + dinutux
Untreated	0.331	0.001	0.009
Dinutux	x	0.001	0.001
aNK	x	x	0.180

CHLA-255-Fluc *P* values

	Dinutux	aNK	aNK + dinutux
Untreated	0.318	0.007	0.012
Dinutux	x	0.055	0.091
aNK	x	x	0.792

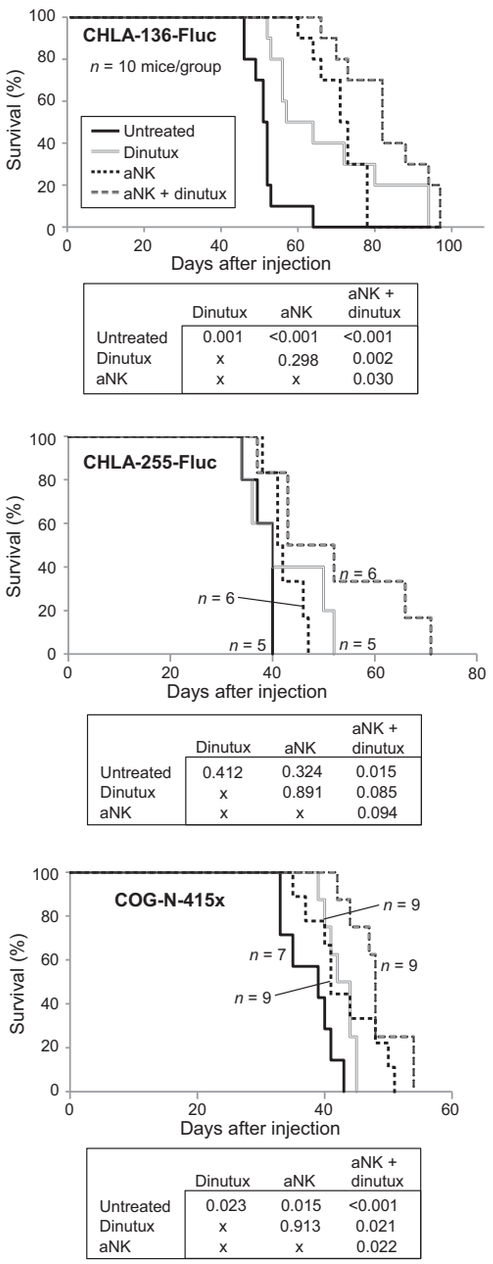
Figure 5.

aNK cells alone and in combination with dinutuximab decrease overall disease burden following primary tumor resection. **A,** Images of disease burden prior to treatment and 2 days after the completion of treatment are shown from mice following resection of primary tumors created by CHLA-136-Fluc and CHLA-255-Fluc cells. **B,** Bioluminescent imaging was performed weekly and tumor flux (photons/sec) measurements were adjusted to baseline observations. AUC was calculated and linear regression was used to assess treatment effects.

invade into tissues (24, 25). In this regard, aNK cells mediate ADCC against neuroblastoma cells at a relatively low 1:2 E:T cell ratio. Second, neuroblastoma cells that survive aNK cell-mediated ADCC may have a decreased ability to invade tissues as we have shown that their invasion into Matrigel *in vitro* is impaired. However, the relative contributions of these and other possible cellular and molecular mechanisms to eradication of residual disease are not currently known.

Although decreased disseminated growth and improved survival could be because of reduction of functionally invasive neuroblastoma cells, tumor cells may already have become established within the liver and/or bone marrow at the time of surgical resection. If so, immunotherapy would need to be effective against disease in these sites, which requires localization and antitumor cell functions of dinutuximab and aNK cells within potentially immunosuppressive microenvironments. Indeed, using these same models in NSG mice without resection, we have found that TGFβ1, which is produced by neuroblastoma cells, inhibits function of aNK cells together with dinutuximab in the tumor microenvironment (17). Thus, targeting immunosuppressive activities with agents such as galunisertib, which inhibits signaling induced by TGFβ1, could further improve antibody-mediated immunotherapy of neuroblastoma cells that disseminated before surgical resection.

We demonstrated that dinutuximab combined with adoptively transferred human aNK cells following surgical resection is consistently effective in improving survival of mice using two human neuroblastoma cell lines and a PDX. Notably, aNK cells alone improved survival for mice with two of the three neuroblastoma xenografts, suggesting that their interaction with ligands on neuroblastoma cells can result in cytotoxicity. The neuroblastoma xenografts used in these experiments are representative of high-risk disease as they were derived from extremely aggressive and drug-resistant metastatic tumors. The CHLA-136 cell line is *MYCN*-amplified while the CHLA-255 cell line overexpresses c-Myc protein, both of which are indicative of a poor prognosis



	Dinutux	aNK	aNK + dinutux
Untreated	0.001	<0.001	<0.001
Dinutux	x	0.298	0.002
aNK	x	x	0.030

	Dinutux	aNK	aNK + dinutux
Untreated	0.412	0.324	0.015
Dinutux	x	0.891	0.085
aNK	x	x	0.094

	Dinutux	aNK	aNK + dinutux
Untreated	0.023	0.015	<0.001
Dinutux	x	0.913	0.021
aNK	x	x	0.022

Figure 6.

aNK cells and dinutuximab improve survival following surgical resection of primary tumors created from CHLA-136-Fluc, CHLA-255-Fluc, and COG-N-415x. Survival plots shown here and the differences in survival between groups were analyzed with linear regression.

(20, 26). The PDX COG-N-415x cells have *MYCN*-amplification and an *ALK* mutation, both of which contribute to aggressive tumor behavior (27). In our experiments, mice were treated with dinutuximab, which is currently a standard-of-care after myeloablative therapy for patients with high-risk neuroblastoma. aNK cells are propagated and activated *ex vivo* similarly to open and upcoming clinical trials (NCT03420963, NCT01853358, NCT03242603, NCT01947322, and NCT02763475). Thus, our model of surgical resection followed by immunotherapy is relevant to the treatment of a spectrum of high-risk neuroblastomas in patients.

Although our experiments mimic immunotherapy in patients with high-risk neuroblastoma, they have possible limitations. First, aNK and neuroblastoma cells were from unrelated donors and so potentially do not provide models for the binding of human leukocyte antigen (HLA) class I molecules by autologous inhibitory and activating killer cell immunoglobulin-like receptors (KIR; ref. 28). However, aNK cells generated with K562-mbIL21 stimulator cells are not inhibited by autologous KIR–ligand interaction (22). Another possible limitation is absence of effector T cells. However, neuroblastoma cells express little or no MHC class I molecules to present peptides to effector T cells and have few gene mutations to generate unique tumor-associated antigens/peptides that could be targets for cytotoxic T cells (29–31). Evidence has not been obtained to date showing an adaptive T-cell immune response to human neuroblastoma (32). These potential limitations could be evaluated in fully immunocompetent mice with transgenic or transplantable syngeneic neuroblastoma tumors that express GD2 or other target antigens for antibodies and that have the spectrum of oncogenic abnormalities associated with aggressive tumor behavior in humans, but such models are not currently available.

In summary, we show that dinutuximab combined with aNK cells mediates ADCC against neuroblastoma cells and inhibits their invasion *in vitro*. Importantly, we demonstrate that mouse survival after surgical resection of primary tumors formed by aggressive human neuroblastoma cells is improved by adoptive transfer of human aNK cells combined with dinutuximab. These data provide support for clinical testing of NK cells that are activated *ex vivo* or *in vivo* and combined with dinutuximab for treatment of residual disease after surgical resection of the primary tumor in children with high-risk neuroblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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