Growth and Activation of Natural Killer Cells Ex Vivo from Children with Neuroblastoma for Adoptive Cell Therapy

Yin Liu\textsuperscript{1,3}, Hong-Wei Wu\textsuperscript{1}, Michael A. Sheard\textsuperscript{1}, Richard Sposto\textsuperscript{1,2}, Srinivas S. Somanchi\textsuperscript{4}, Laurence J.N. Cooper\textsuperscript{4}, Dean A. Lee\textsuperscript{5}, and Robert C. Seeger\textsuperscript{1}


division of Pediatrics, M.D. Anderson Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai, China; and 4Division of Pediatrics, MD Anderson Children's Medical Center, University of Texas, Houston, Texas

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Corresponding Author: D.A. Lee and R.C. Seeger are co-senior authors of this article.

Introduction

Although outcome has steadily improved over the past 20 years for patients with high-risk, metastatic neuroblastoma, long-term event-free survival (EFS) is still only 45% (1–3).

Purpose: Adoptive transfer of natural killer (NK) cells combined with tumor-specific monoclonal antibodies (mAb) has therapeutic potential for malignancies. We determined if large numbers of activated NK (aNK) cells can be grown ex vivo from peripheral blood mononuclear cells (PBMC) of children with high-risk neuroblastoma using artificial antigen-presenting cells (aAPC).

Experimental Design: Irradiated K562-derived Clone 9.mblL21 aAPC were cocultured with PBMC, and propagated NK cells were characterized by flow cytometry, cytotoxicity assays, Luminex cytokine:assays, and a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse model of disseminated neuroblastoma.

Results: Coculturing patient PBMC with aAPC for 14 days induced a 2,363- to 443-fold expansion of CD56\textsuperscript{+}CD3\textsuperscript{−}CD14\textsuperscript{−}NK cells with 83\% ± 3\% purity (n = 10). Results were similar to PBMC from normal donors (n = 5). Expression of DNAM-1, NKGD2, FcyRIII/CD16, and CD56 increased 6- ± 3-, 10- ± 2-, 21- ± 10-, and 18- ± 3-fold, respectively, on day 14 compared with day 0, showing activation of NK cells. In vitro, aNK cells were highly cytotoxic against neuroblastoma cell lines and killing was enhanced with GD2-specific mAb ch14.18. When mediating cytotoxicity with ch14.18, release of TNF-α, granulocyte macrophage colony-stimulating factor, IFN-γ, sCD40L, CCL2/MCP-1, CXCL9/MIG, and CXCL11/ITAC by aNK cells increased 4-, 5-, 6-, 15-, 265-, 917-, and 363-fold (151–9,121 pg/mL), respectively, compared with aNK cells alone. Survival of NOD/SCID mice bearing disseminated neuroblastoma improved when treated with thawed and immediately intravenously infused cryopreserved aNK cells compared with untreated mice and was further improved when ch14.18 was added.

Conclusion: Propagation of large numbers of aNK cells that maintain potent antineuroblastoma activities when cryopreserved supports clinical testing of adoptive cell therapy with ch14.18. Clin Cancer Res; 19(8); 1–12. ©2013 AACR.

Addition of immunotherapy with antitumor cell disialo-ganglioside (GD2) monoclonal antibody (mAb) ch14.18 along with interleukin (IL)-2 and granulocyte macrophage colony-stimulating factor (GM-CSF) to 13-cis retinoic acid improves EFS and overall survival for children who have a clinical response after induction chemotherapy and myeloablative consolidation therapy (3). However, 40% of patients still develop disease progression or relapse during or after immunotherapy (3).

mAb immunotherapy for residual disease may be further enhanced by improving the ability of natural killer (NK) cells to function in antibody-dependent cell-mediated cytotoxicity (ADCC) and to secrete antitumor cytokines and chemokines. Strategies include modifying mAbs to have high affinity interaction with NK cell FcRRII/CD16 or to deliver cytokines via mAb–cytokine fusion proteins to the tumor microenvironment (4), enhancing activation of NK cells in vivo using immune-modulating drugs, such as lenalidomide (5, 6), or growing and activating NK cells ex vivo for adoptive cell therapy. Some of these approaches also may be combined with cytotoxic chemotherapy or
targeted therapy for more effective treatment of measurable disease.

Adoptive cell therapy with NK cells alone or combined with mAbs has therapeutic potential for a wide variety of human malignancies, including neuroblastoma (7). One approach for obtaining NK cells has been to harvest large numbers of peripheral blood lymphocytes by leukapheresis, deplete allogeneic T cells, and activate the remaining NK cells with IL-2 before reinfusion. In this manner, haploidentical NK cell therapy for acute myelogenous leukemia (16) and therapy for glioblastoma (10–14), but clinical testing of such NK cell therapy has been limited because of the inability to obtain large numbers of pure NK cells that do not senesce after replication (15, 16).

We recently genetically engineered K562 cells that coexpress CD64/FcγRI, CD86/B7-2, CD137L/MIC, and truncated CD19, and membrane-bound IL-21 (K562 Clone 9.mbl21) to serve as artificial antigen-presenting cells (aAPC) promoting sustained ex vivo proliferation of human NK cells (17, 18). The responding NK cells have a significant increase in telomere length compared with freshly isolated NK cells, which may explain their sustained proliferation (18). With this method, large numbers of activated NK cells (aNK) can be generated from normal adult donors with high purity and functionality.

In this study, we show that K562 Clone 9.mbl21 cells allow the generation of large numbers of NK cells exhibiting activation characteristics from peripheral blood mononuclear cells (PBMC) of children with high-risk neuroblastoma. These aNK cells are highly cytotoxic alone or with mAb ch14.18 against multidrug sensitive and resistant neuroblastoma cell lines in vitro and secrete an array of cytokines and chemokines with antitumor potential while mediating ADCC. These aNK cells maintain their functional activities after viable cryopreservation, and most importantly, retain potent antitumor activity with ch14.18 when intravenously infused immediately after thawing into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice with disseminated human neuroblastoma.

Materials and Methods

Cell lines

Neuroblastoma cell lines CHLA-255 and CHLA-136 were maintained in Iscove’s modified Dulbecco’s medium (IMDM) with 20% FBS (Invitrogen), and LA-N-1 was maintained in RPMI-1640 (Mediatech) with 10% FBS. CHLA-255-Fluc cells were transduced with the firefly luciferase (Fluc) gene (CHLA-255-Fluc) using a lentivirus vector (19). CHLA-255-Fluc is sensitive to etoposide and melphalan in vitro, whereas CHLA-136 and LA-N-1 are resistant to etoposide and melphalan [resistance: IC50 > 1,000 ng/mL and >10,000 ng/mL for etoposide and melphalan, respectively; Dr. Nino Keshelava (Division of Hematology/Oncology, Keck School of Medicine, University of Southern California, Los Angeles, CA), personal communication and refs. 20–22]. The K562 Clone 9.mbl21 cell line was grown in RPMI-1640 with 10% FBS (17, 18).

Preparation of peripheral blood mononuclear cells

Peripheral blood was obtained from 10 patients with high-risk neuroblastoma and 5 healthy adults, and PBMC were isolated by density separation using Histopaque-1077 (Sigma-Aldrich; ref. 23). Written informed consent was obtained from healthy donors in accordance with a protocol approved by the Committee on Clinical Investigation at Children’s Hospital Los Angeles (Los Angeles, CA) for the use of cells for cancer and/or blood research. Anonymous specimens from patients with high-risk, stage IV (metastatic) neuroblastoma were obtained from patients enrolled and consented in therapeutic and biology protocols of the Children’s Oncology Group (COG).

NK cell propagation and activation

K562 Clone 9.mbl21 cells (clinical-grade master cell bank designated CILK CT64.86.41BBL.CD19. mbl21) were derived from Clone 9 cells (generated with Dr. Carl June, University of Pennsylvania, Philadelphia, PA) at MD Anderson Cancer Center (Houston, TX) using the Sleeping Beauty transposon/transposase system to express a membrane-bound variant of IL-21 (18). Before initiating cocultures of K562 Clone 9.mbl21 aAPC and PBMC on day 0, the aAPC were irradiated with 100 Gy using a gamma irradiator, washed with PBS, and resuspended in NK cell expansion medium (NKEM) containing RPMI-1640 and 10% FBS with 50 IU/mL recombinant human IL-2.

Translational Relevance

Adoptive cell therapy with natural killer (NK) cells has therapeutic potential for malignancies. We report highly efficient ex vivo numeric growth and activation of NK cells (aNK) from blood of patients with neuroblastoma. K562-derived artificial antigen-presenting cells designated as Clone 9.mbIL21 act as feeder cells to stimulate NK cells to proliferate more than 2,000-fold in 14 days and to become highly cytotoxic against multidrug sensitive and resistant neuroblastoma cell lines when alone or when combined with anti-GD2 antibody ch14.18. Incubation of aNK cells and ch14.18 with neuroblastoma cells markedly increased secretion of TNF-α, granulocyte macrophage colony-stimulating factor, IFN-γ, sCD40L, CCL2/MCP-1, CXCL9/MIG, and CXCL11/L-TAC. Cryopreserved aNK cells that were infused intravenously immediately after thawing into nonobese diabetic/severe combined immunodeficient mice bearing disseminated neuroblastoma significantly decreased tumor growth and increased mouse survival, especially when combined with ch14.18. These results support clinical testing of ex vivo grown and activated autologous NK cells combined with ch14.18 as treatment of neuroblastoma.

Adoptive cell therapy with NK cells alone or combined with mAbs has therapeutic potential for a wide variety of human malignancies, including neuroblastoma (7). One approach for obtaining NK cells has been to harvest large numbers of peripheral blood lymphocytes by leukapheresis, deplete allogeneic T cells, and activate the remaining NK cells with IL-2 before reinfusion. In this manner, haploidentical NK cell therapy for acute myelogenous leukemia attained remission in poor-prognosis adults (8) and main-
PBMC (10^6) from patients with neuroblastoma were incubated at 4°C for 1 hour with aAPC (2.5 × 10^6) in T25 flasks (Corning, 25 cm²), whereas PBMC (10^6) from patients with neuroblastoma were incubated with aAPC (0.5 × 10^6) in 6-well tissue culture plates (Corning, 9.5 cm²), both in NKEM at a total cell concentration of 0.5 × 10^6/mL. An equal-volume of fresh NKEM was added on day 3. At day 7 of coculture, cells were counted, new irradiated aAPC were added (total cell:aAPC ratio = 2:1), and cells were seeded into T75 or T150 flasks with additional NKEM (total cell concentration ≤0.5 × 10^6/mL). An equal-volume of fresh NKEM was added on day 11. Cells were grown for 14 days, at which time they were phenotyped by flow cytometry and tested for cytotoxicity. The remainder were aliquoted and viably frozen in a mixture of 50% Cryoprotective Medium (Lonza), 25% RPMI-1640, and 25% FBS.

Flow cytometry
Surface marker staining was conducted as previously described (24). Briefly, cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (PBS with 0.1% NaN₃ and 0.1% bovine serum albumin) and centrifuged for 10 minutes at 400 × g. Antibodies listed in Supplementary Table S1 were added in the dark at 4°C using concentrations previously determined by titration. Isotype-matched irrelevant mAbs were used to define nonspecific staining. Cells were incubated at 4°C for 90 minutes and washed twice in FACS buffer. Flow cytometry analysis was conducted using a BD LSR II flow cytometer, DIVA software (BD Biosciences) and Flowjo analysis software (Tree Star). The mean fluorescence intensity (MFI) ratio was calculated as follows: MFI of viable cells stained with specific antibody/MFI of viable cells stained with an isotype-matched irrelevant antibody.

In vitro cytotoxicity assays
NK cells that had been grown for 14 days were seeded into 96-well Costar black tissue culture plates (Corning) in 0.1 mL RPMI-1640 with 50 IU/mL IL-2 and 2% FBS. Three neuroblastoma cell lines (LA-N-1, CHLA-136, and CHLA-255-Fluc) were labeled with calcein-acetoxymerthyl (calcein-AM; 5 μg/10⁶ cells) for 30 minutes (25). Neuroblastoma cells were added to aNK cells at various effector-to-target (E:T) ratios in IMDM for CHLA-255-Fluc and CHLA-136 or RPMI-1640 for LA-N-1 with 2% FBS and without or with anti-GD2 mAb ch14.18 (0.1 μg/mL; provided by the National Cancer Institute, Frederick, MD). Cells were then coincubated for 6 hours at 37°C, and live cells, which contain calcein-AM, were quantified with digital imaging microscopy system (DIMSCAN) as previously described (25).

Cytometric bead array and Luminex assays
NK cells were grown for 14 days, cryopreserved, thawed, and cultured in NKEM for 72 hours before generating conditioned media by coculturing aNK cells with CHLA-255-Fluc or CHLA-136 tumor cell lines (aNK:tumor cells = 1:1) without or with ch14.18 (0.1 μg/mL) for 24 hours. Conditioned media were examined for granzymes A and B using the cytometric bead array (CBA) assay from BD Biosciences and for cytokines and chemokines using the 39-, 23-, and 9-plex Human Cytokine/Chemokine Panels from Millipore according to the manufacturer's protocols. For granzyme analyses, data were collected on an LSR II flow cytometer using DIVA software (BD Biosciences). FCAP software (BD Biosciences) was used to fit standard curves to the data obtained from the analyte standards and to calculate absolute concentration values for each analyte from its respective standard curve. In the Luminex assay, data were acquired with a Luminex-200 instrument (Luminex Corporation). Cytokine concentrations were determined by referring to a standard curve and expressed as pg/mL using xPonent software (Luminex Corporation).

Murine model of disseminated neuroblastoma
NOD/SCID mice were purchased from the Jackson Laboratory. Rat anti-mouse CD122 (200 μg/mouse) was injected intraperitoneal 1 day before tumor cell injection and then every other week to eliminate residual murine NK cells. CHLA-255-Fluc cells were injected intravenously on day 0. Multiple intravenous injections of expanded aNK cells were given together with intravenous IL-2 and without or with ch14.18 as described in Results and figure legends. Tumor growth was assessed weekly by bioluminescence imaging 15 minutes after intraperitoneal injection of a-luciferin potassium salt solution (1.5 mg/mouse) using a Xenogen IVIS-200 system (Caliper Life Sciences). Photons emitted were quantified with the Living Image 3.0 software (Caliper Life Sciences). Animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Usage Committee of Children's Hospital Los Angeles.

Statistical analysis
Data were analyzed using the statistical software Stata (version 11) and are represented as mean ± SD unless otherwise stated. ANOVA was conducted to determine the significance of observed differences. Mouse survival time was defined as the length of time (in days) from the tumor injection date until the end of the study or time of sacrifice due to disease progression. Censored normal regression was used to examine whether any difference in survival time existed because of varying treatments. The censored Wilcoxon test was used to examine the difference in the survival curves among the different treatment groups. A P value of less than 0.05 was considered statistically significant.

Results
Propagation of NK cells
PBMC from 10 children with high-risk neuroblastoma and from 5 normal adults were cultured with K562-derived aAPC and IL-2 (Fig. 1). Total cell number in cultures of PBMC from 10 patients with neuroblastoma increased by a mean of 116-fold (range, 41- to -200-fold), and CD56⁺ CD3− NK cells increased by a mean of 2,363-fold (range,
600- to 6,362-fold) by day 14 of coculture. This growth was similar in cultures from 5 healthy adult donors, with a mean of 126-fold (range, 77–175) increase in total cells and 2,593-fold (range, 1,051–5,606) increase in NK cells (Fig. 1A). The doubling time for NK cells from patients was 1.24 days and from normal donors was 1.25 days. Propagation of the effector cells could be prolonged for at least 28 days with an additional 40-fold increase in total cell number compared with day 14 (data not shown). Final cultures from patients had an average of 83.2% ± 2.8% CD56+CD3−CD14− NK cells and 9.1% ± 2.2% CD3+ T cells of which 6.3% ± 2.1% were TCR-γδ+ T cells. For normal donors, the final product had an average of 76.6% ± 3.4% CD56+CD3−NK cells and 19.4% ± 2.5% CD3+ T cells (TCR-γδ+ T cells were not evaluated; Fig. 1B–D).

The ability of K562-derived aAPC to selectively propagate NK cells was shown by analyses of the hematopoietic cell subpopulations on day 0 and then on day 14 of coculture. NK-cell frequency in PBMC from patients and normal donors on day 0 was similar at 4.7% ± 0.5% and 4.7% ± 0.9%, respectively. Differences between groups were observed for CD3+ T cells (36.9% ± 6.9% for patients and 63.4% ± 5.2% for normal donors) and CD14+ monocytes (27.6% ± 7.2% for patients and 14.5% ± 3.7% for normal donors; P < 0.0001; Fig. 1D). Specimens from patients were anonymous, and so it is not possible to correlate clinical variables such as disease status and treatment with PBMC subsets. At day 14, large decreases in CD3+ and CD14+ cells were observed in both groups (Fig. 1D). Additional analyses conducted only on cells from patients showed a decrease in CD4+CD3− T cells, CD8+CD3− T cells, CD4−CD25−CD3− T cells, 6B11+CD3− invariant NKT cells, CD14+ monocytes, and CD19+ B lymphocytes at day 14 (0.2% ± 0.2%, 2.4% ± 0.4%, 0.05% ± 0.02%, 0.07% ± 0.01%, 0.2% ± 0.1%, and 0.6% ± 0.4%, respectively) compared with day 0 (24.0% ± 5.3%, 12.1% ± 3.1%, 0.2% ± 0.1%, 0.2% ± 0.1%, 27.6% ± 7.2%, and 19.9% ± 3.2%, respectively). The difference in the cell frequency of all cell types over time was significant (P < 0.01).

Expression of cell surface immune-function markers by aNK cells

Expression of natural cytotoxicity receptors DNAM-1, NKG2D, and Nkp46, the degranulation marker CD107a/LAMP1, the adhesion molecule CD56, the chemokine receptor CXCR4, and Fc receptors CD16, CD32, and CD64 was quantified by flow cytometry for NK cells from 10 patients before (day 0) and after K562-aAPC–stimulated expansion (day 14; Fig. 2). The MF ratio for DNAM-1, NKG2D, CD16, and CD56 increased by 6.2- ± 3.2-, 10.3- ± 2.4-, 20.9- ± 19.7-, and 17.8- ± 2.9-fold, respectively. On average, there was little or no difference in Nkp46, CD107a, CXCR4, CD32, and CD64 expression between NK cells at days 0 and 14.
Direct cytotoxicity and ADCC by expanded aNK cells

The cytotoxicity of aNK cells from 10 patients with neuroblastoma and 5 normal donors was tested against the neuroblastoma cell lines CHLA-255-Fluc (drug sensitive), LA-N-1 (multidrug resistant), and CHLA-136 (multidrug resistant; refs. 20–22) after a 6-hour incubation using the calcein-AM assay (Fig. 3; ref. 25). Both multidrug-sensitive and -resistant cell lines were sensitive to aNK cell direct cytotoxicity and to ch14.18-mediated ADCC with significant killing occurring at 1:1, 1:2, and 1:5 aNK:neuroblastoma cell ratios ($P < 0.001$). aNK cell cytotoxicity was greater against all 3 tumor cell lines when mediating ADCC with ch14.18 ($P < 0.05$ for all). Cytotoxicity mediated by aNK cells from patients and normal donors was similar.

Cytokine secretion and release of granzymes A and B by aNK cells during direct cytotoxicity and ADCC

To further determine the potential antitumor effects of aNK cells, we evaluated the release of 71 cytokines and of granzymes A and B by coculturing aNK cells from 5 patients with CHLA-255-Fluc (drug sensitive) or CHLA-136 (multidrug resistant) neuroblastoma cells without or with ch14.18 for 24 hours (Fig. 4). In the absence of ch14.18, coculture of aNK cells and CHLA-255-Fluc or CHLA-136 cells significantly increased 56 and 32 cytokines in the culture media, respectively, compared with aNK cells alone (Fig. 4). Notably, patient aNK cells cocultured with CHLA-255-Fluc or CHLA-136 with ch14.18 increased the release of TNF-α (4- and 5-fold), GM-CSF (5- and 7-fold), IFN-γ (6-fold for each cell line), CCL2/MCP-1 (265- and 13-fold), CXCL9/MIG (917- and 100-fold), CXCL11/I-TAC (363- and 35-fold), FGF2 (9- and 12-fold), and sCD40L (15- and 11-fold) compared with aNK cells alone (Fig. 4). In similar experiments, coculture of aNK cells from 5 normal donors with CHLA-255-Fluc cells without or with ch14.18 significantly increased release of 19 and 11 cytokines, respectively, compared with aNK cells alone (Supplementary Fig. S1). Cytokines and chemokines for which at least one half of the tests were below the level of detection were excluded from these analyses (see listing in Supplementary Table S2). Tumor cells alone or combined with ch14.18 produced a background cytokine/chemokine level of less than 10 pg/mL for each analyte. High levels of granzyme A and B were released by aNK cells from patients and normal donors but were not different for aNK cells alone versus aNK cells combined with tumor cells without or with ch14.18 (Fig. 4 and Supplementary Fig. S1). Thus, the interaction of aNK cells with neuroblastoma cells significantly affected the release of potential antitumor cytokines.
Antitumor activity of cryopreserved aNK cells in a NOD/SCID mouse model of disseminated neuroblastoma

The antitumor activity of cryopreserved aNK cells grown with K562-mbIL21.aAPC was tested in vivo using a model of disseminated neuroblastoma in which CHLA-255-Fluc cells are injected intravenously into NOD/SCID mice. Bioluminescent imaging of untreated mice does not detect disease at 7 days but does so in at least 50% at 21 days and 100% at 28 days, and so treatments were begun at 7 or 21 days to model different levels of tumor burden.

Initial experiments compared aNK cells from single normal donors that were cryopreserved, thawed and either cultured for 3 days before injection, or thawed and immediately injected intravenously (Fig. 5). Beginning at 7 days, mice were treated weekly for 4 weeks with aNK cells alone or in combination with ch14.18 (10⁷ aNK cells/mouse ×/wk, 3 μg IL-2/mouse 2×/wk, and 15 μg ch14.18/mouse 2×/wk). Tumor growth was reduced and mouse survival was longer among mice receiving any treatment compared with untreated mice. Mice receiving treatment that included ch14.18 had an increased survival time compared with those receiving aNK cells alone (P = 0.01). Mice receiving thawed and immediately injected or thawed and cultured aNK cells with ch14.18 had similar tumor growth (P = 0.26) and survival (P = 0.73). A second experiment using aNK cells from another normal donor with the same schedules and doses of aNK cells, IL-2, and ch14.18 confirmed no difference in efficacy between thawed and cultured versus thawed and immediately injected aNK cells (Supplementary Fig. S2). These results show that cryopreserved aNK cells infused immediately after thawing retain their antitumor functions.

The next experiment compared the impact of frequency and duration of aNK treatment using cells from a single patient donor that were cryopreserved, thawed, and then immediately injected intravenously (Supplementary Fig. S3). aNK cells (10⁷) were injected twice weekly × 3 weeks (group 2, aNK alone and group 3, aNK with ch14.18) or once weekly × 6 weeks (group 4, aNK with ch14.18) beginning at day 7. IL-2 (3 μg/mouse, 4× or 2×/wk) and ch14.18 (15 μg/mouse, 4× or 2×/wk) were given in the same weeks as aNK cell infusions. Tumor growth in untreated mice was significantly greater than that of all 3 treated groups (P < 0.001; Supplementary Fig. S3B). Tumor growth of treatment groups with or without ch14.18 also was significantly different (P < 0.001). Tumor growth of treatment groups receiving 2×/wk or 1×/wk aNK with ch14.18 was significantly different up to day 62 after tumor cell injection (P = 0.006) but not afterwards (P = 0.49), and so, overall there was no difference (P = 0.10). For mice treated with aNK and ch14.18, 3 of 10 in the 2×/wk group and 1 of 10 in the 1×/wk group had no detectable tumor by imaging at day 83 (55 and 34 days after the last treatment). With respect to survival, the untreated group was significantly worse than all treatment groups (P < 0.0001). Survival of the 2 groups receiving aNK and ch14.18 was significantly better than that of the group receiving aNK alone (P = 0.0001 and P = 0.0002; Supplementary Fig. S3C). Survival of mice
Natural Killer Cell Immunotherapy for Neuroblastoma

Figure 4. Cytokine and chemokine release from K562 Clone 9.mbl21 A2PC-expanded aNK cells after a 24-hour incubation with neuroblastoma cell lines CHLA-255-Fluc and CHLA-136 alone or with anti-GD2 antibody ch14.18. Day 14 expanded aNK cells from 5 patient donors with neuroblastoma were thawed and cultured for 72 hours with 50 IU/mL IL-2 before coculture with CHLA-255-Fluc or CHLA-136 cells (1:1 E:T ratio, 24 hours) without or with ch14.18, and then supernatants were collected for both Luminex and the CBA assays. Means and SD of fold changes are shown in dots and lines, respectively; red square dots indicate significant P values (P < 0.05) for each comparison versus aNK cells alone. Fold changes and concentration (pg/mL) of cytokines or chemokines secreted by aNK cells exposed to different conditions (numerator in each function) are shown on the right-hand side of each data point.
receiving aNK cells and ch14.18 2 ×/wk was better than that of mice receiving aNK and ch14.18 1 ×/wk (P = 0.038). These results confirm in vivo the antineuroblastoma activity of aNK cells that were cryopreserved, thawed, and then immediately infused with ch14.18 and suggest a modest effect of treatment schedule.

The last experiment compared different treatments (aNK alone, ch14.18 alone, and aNK combined with ch14.18) beginning at day 7 or 21 when disease was not or was detectible by imaging (Fig. 6 and Supplementary Fig. S4). Groups of mice received cryopreserved aNK cells (10⁷) from a single normal donor PBMC twice weekly for 4 weeks with IL-2 (3 μg/mouse, 2 ×/wk) and with or without ch14.18 (15 μg/mouse, 2 ×/wk). Two other groups received ch14.18 alone in the same schedule and dose. Beginning treatment at day 7 with NK cells alone was associated with decreased tumor growth (P = 0.007) and increased survival (P = 0.032) when compared with untreated control mice (Fig. 6). Treatment with ch14.18 alone caused a further decrease in tumor growth (P < 0.001) and increase in survival (P < 0.001).
Tumor growth was most reduced and survival most increased when treatment included both ch14.18 and NK cells, and this combination was more effective than either NK cells ($P < 0.001$) or ch14.18 ($P < 0.001$) alone. Even when treatment was begun 21 days after tumor cell injection (Supplementary Fig. S4), when disseminated disease was visualized in all mice, tumor growth was less after 2 weeks of treatment with NK and ch14.18 together than after no treatment ($P < 0.001$), NK cells alone ($P = 0.003$), or ch14.18 alone ($P = 0.039$). Survival was greater after treatment with NK and ch14.18 than no treatment ($P = 0.004$), marginally better than NK cells alone ($P = 0.086$), and equivalent to ch14.18 alone ($P = 0.297$). All treatments were more effective when begun on day 7 compared with day 21 (two-way ANOVA; $P = 0.009$), and tumor growth was inhibited by the combination of aNK with ch14.18 regardless of when treatment was initiated.

**Discussion**

Repeated infusions of aNK cells and antitumor antibodies may provide an effective strategy for treating minimal residual disease and possibly measurable disease when combined with cytotoxic therapy. Somanchi and colleagues...
and Denman and colleagues reported a new method to efficiently grow large numbers of aNK cells ex vivo using K562 Clone 9.mbIL21 cells as aAPC (17, 18). In their study, the number of NK cells from PBMC of normal donors increased by a mean of 47,967-fold in 21 days, had a marked increase in telomere length after stimulation and did not senesce, even after 6 weeks of culture (17, 18). Using the same method, we show for the first time that large numbers of aNK cells can be grown from PBMC of patients with high-risk neuroblastoma. These aNK cells alone and with anti-GD2 mAb ch14.18 are highly cytotoxic and secrete multiple cytokines with antitumor potential when cultured with multidrug sensitive and resistant neuroblastoma cell lines. Importantly, these aNK cells retain antitumor function(s) in vivo after cryopreservation. These results provide a model for clinical testing of adoptive cell therapy with activated autologous NK cells and anti-GD2 mAb ch14.18.

While a number of strategies are possible for generating human NK cells for adoptive cell therapy, the low cell number available for adoptive transfer has limited clinical testing of this immunotherapeutic strategy (7). Leukapheresis, T-cell depletion, and short-term culture in IL-2 can provide haploidentical or autologous NK cells but rarely in sufficient quantity for more than a single infusion (8, 9, 26, 27). Culture of T-cell–depleted products from patients with melanoma for approximately 21 days in IL-2 resulted in 278- to 1,097-fold expansion of autologous NK cells, and reinfused NK cells were shown to circulate for at least 1 week without causing tumor regression (14). In another clinical study, allogeneic NK cells from related donors were expanded ex vivo 3- to 131-fold with hydrocortisone and IL-15 and infused to treat patients with advanced non–small cell lung cancer (28). Other methods for ex vivo expansion have been reported from preclinical studies. NK cells from normal donors cultured in defined medium with IL-2 expanded a median of 193-fold (range, 21- to 277-fold; ref. 29) and from patients with multiple myeloma a mean of 1,625-fold (range, 502- to 2,658-fold; ref. 30). A mixture of IL-7, recombinant human stem cell factor, IL-2, and IL-15 in defined medium stimulated a 3-log increase in NK cells from cord blood (11). aAPC have been genetically engineered and used with or without additional cytokines to stimulate expansion. Addition of IL-2 to K562 cells that were modified to express membrane-bound IL-15 and 41BB ligand stimulated a mean of 277-fold increase (range, 201- to 1,459-fold) in NK cells from normal donors and a similar level from patients with acute leukemia (12). KT64.41BB.LA2 cells, which naturally express IL15Rα and MICA/B, stimulated a 1,000-fold increase of NK cells from blood of normal donors (13). K562-MICA-41BB.LA2 IL-15 cells stimulated the expansion of NK cells by a mean of 550-fold (range, 201-880; ref. 31).

Using K562 Clone 9.mbIL21 cells and added IL-2 (17, 18), we show a mean of 2,363-fold (range, 600- to 6,362-fold) expansion of NK cells from patients with high-risk neuroblastoma at day 14, with 83% of the total cells being CD3ε− CD3ε− NK cells. In contrast to other methods, telomere length in NK cells significantly increases as compared with fresh NK cells with this method (18), which allows culturing for at least 28 days to obtain an even greater number of aNK cells. This positive effect on telomere length is likely due to IL-21 activation of STAT3 (32) that in turn regulates human telomerase reverse transcriptase (hTERT) expression (33). Importantly, growth of NK cells from patients and normal donors was equivalent, which strongly supports the feasibility of preparing autologous aNK cells for therapy. Without T-cell depletion, less than 20% of the total population at day 14 was T cells, and most of these were TCR-γδ T cells. Because K562 Clone 9.mbIL21 cells are lethally irradiated before culture and are lysed by the expanding NK cells, the risk of infusing viable K562 Clone 9.mbIL21 cells to patients is negligible. Thus, we anticipate that it will be possible to generate billions of aNK cells from peripheral blood of children with neuroblastoma for multiple infusion treatments, without the need for apheresis.

Generation of activated CD56+CD3− NK cells from PBMC of both patients and normal donors with K562 Clone 9.mbIL21 cells and IL-2 is highly reproducible. More than 80% of cells are CD56+ CD3− NK cells, and they display an activated phenotype as shown by more than a 6-fold increase in the MFI ratio for NKG2D, DNAM-1, CD16, and CD56 at day 14 compared with day 0. NK cells generated from PBMC of patient and normal donors are highly cytotoxic against chemotherapy sensitive and resistant neuroblastoma cell lines when alone and even more so with mAb ch14.18. Remarkably, only approximately 20% of tumor cells survived ADCC at a 1:1 aNK-tumor cell ratio after 6 hours. Our previous study showed increased expression of inhibitory killer cell immunoglobulin-like receptor (KIR) KIR2DL2/3 but not of inhibitory KIR2DL1, NKG2A, or NKG2C on expanded compared with fresh NK cells (18). NKG2A was expressed by 85% of expanded NK cells but the other receptors were expressed by only 15% to 30%. Expanded NK cells killed 721.221 cell line targets expressing HLA group C1 or Bw4, which are ligands for inhibitory KIR2DL2/3 or KIR3DL1, respectively, equally as well as the parent cell line without these ligands (18). Altogether, our results are similar to earlier reports with IL-2 alone or in combination with other cytokines, except that a much larger number of NK cells are obtained with the K562 Clone 9.mbIL21 model (11, 12, 34).

We showed that a complex array of cytokines and chemokines are released in vitro upon aNK cell interaction with drug-sensitive and -resistant tumor cell lines, especially during ADCC mediated by ch14.18. Although it is not possible to relate these data to what might occur in vivo, on balance, the pattern suggests an antitumor effect. Levels of TNF-α, GM-CSF, IFN-γ, sCD40L, CCL2/MCP-1, CXCL9/MIG, and CXCL11/T-AC increased 4-, 5-, 6-, 15-, 265-, and 363-fold with concentrations ranging from 151 to 9,121 pg/mL. However, cytokines that are important for NK cell proliferation and activation, IL-12p40, IL-12p70, and IL-15 were present at low levels (<25 pg/mL). IFN-γ induces CXCL9 and CXCL11 that recruit T cells and NK cells and have angiostatic properties (35). CD40 ligand (CD40L) is essential for the initiation of antigen-specific T-cell
responses, and activation of the CD40 pathway by sCD40L may contribute to antitumor immune responses. sCD40L also may have direct effects upon tumor cell proliferation and survival (36, 37). Because the increased release of these cytokines was only achieved when aNK cells interacted with neuroblastoma cells, especially via mAb ch14.18, quantification of cytokines in blood may provide useful indicators of aNK-tumor cell interactions in vivo.

The use of autologous aNK cells for expansion will likely prevent host reactions against the adoptively transferred cells that may occur with allogeneic aNK cells, potentially resulting in impaired survival, migration, and function. Although there is a possibility that autologous aNK antitumor function could be suppressed by KIR inhibitory receptor/HLA class I molecule interactions as has been suggested from reviews of patients undergoing myeloablative therapy and autologous hematopoietic stem cell transplantation (38) and of patients treated with an anti-GD2/IL-2 fusion protein (39), available in vitro data suggest that highly aNK cells are not impacted by these interactions (13, 18). Furthermore, 60% of individuals have NK cells that express KIRs but do not express the cognate HLA class I ligands for the KIRs (missing KIR ligand; refs. 40, 41), and these NK cells can kill neuroblastoma cells with anti-GD2 mAb (42). Finally, neuroblastoma cells often do not express surface HLA class I molecules, the ligands for inhibitory KIR receptors (43–47). To date, the impact of KIR/HLA class I interactions on outcome in patients with neuroblastoma treated with the ch14.18 mAb has not been evaluated (3).

aNK cells that are grown and activated with K562 clone 9. mblL21 cells are active against disseminated human neuroblastoma cells growing in NOD/SCID mice. Viably cryopreserved aNK cells that were thawed and cultured for 3 days or thawed and immediately infused intravenously were equally effective when combined with mAb ch14.18 against neuroblastoma. Treatment with aNK cells alone, ch14.18 alone, or the combination of both was more effective when begun at 7 days after tumor cell injection before disease could be imaged than when begun at 21 days when disseminated disease was readily imaged. In either setting, aNK combined with ch14.18 was more effective than aNK cells or ch14.18 alone.

The K562 Clone 9.mblL21 NK cell growth and activation method provides an important advance for generating aNK cells in high numbers, purity, and functionality from PBMC of patients with neuroblastoma for use in NK cell-based immunotherapy. Because viably cryopreserved aNK can be thawed and immediately infused into patients, it will be feasible to grow and cryopreserve aNK cells in a central laboratory for later shipment to institutions participating in multicenter treatment protocols to evaluate dose and toxicity as well as aNK cell survival, expansion, migration, and, within the context of such studies, antitumor activity. Adoptive cell therapy with aNK combined with ch14.18 may be effective against a relatively small amount of disease but likely will need to be combined with cytotoxic therapy to be effective against a large amount of disease.

Disclosure of Potential Conflicts of Interest
D.A. Lee is the co-owner of InCellerate, receives research support from Celgene, and is a consultant/advisory board member of Celgene Cellular Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Y. Liu, H.-W. Wu, L.J.N. Cooper, D.A. Lee, R.C. Seeger
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Liu, H.-W. Wu, M.A. Sheard, R.C. Seeger
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Liu, H.-W. Wu, R. Sposto, L.J.N. Cooper, R.C. Seeger
Writing, review, and/or revision of the manuscript: Y. Liu, M.A. Sheard, L.J.N. Cooper, D.A. Lee, R.C. Seeger
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Liu, H.-W. Wu, S.S. Somanchi, L.J. N. Cooper, R.C. Seeger
Study supervision: R.C. Seeger

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