Expanded CD56superbright CD16+ NK Cells from Ovarian Cancer Patients Are Cytotoxic against Autologous Tumor in a Patient-Derived Xenograft Murine Model

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Abstract

Natural killer (NK) cells are useful for cancer immunotherapy and have proven clinically effective against hematologic malignancies. However, immunotherapies for poor prognosis solid malignancies, including ovarian cancer, have not been as successful due to immunosuppression by solid tumors. Although rearming patients' own NK cells to treat cancer is an attractive option, success of that strategy is limited by the impaired function of NK cells from cancer patients and by inhibition by self-MHC. In this study, we show that expansion converts healthy donor and immunosuppressed ovarian cancer patient NK cells to a cytotoxic CD56superbright CD16+ subset with activation state and antitumor functions that increase with CD56 brightness. We investigated whether these expanded NK cells may overcome the limitations of autologous NK cell therapy against solid tumors. Peripheral blood- and ascites-derived NK cells from ovarian cancer patients were expanded and then adoptively transferred into cell-line and autologous patient-derived xenograft models of human ovarian cancer. Expanded ovarian cancer patient NK cells reduced the burden of established tumors and prolonged survival. These results suggest that CD56superbright NK cells harbor superior antitumor function compared with CD56dim cells. Thus, NK cell expansion may overcome limitations on autologous NK cell therapy by converting the patient's NK cells to a cytotoxic subset that exerts a therapeutic effect against autologous tumor. These findings suggest that the value of expanded autologous NK cell therapy for ovarian cancer and other solid malignancies should be clinically assessed.

Introduction

Ovarian cancer is a deadly gynecologic cancer with a 5-year survival rate of less than 50% (1). Each year in the United States, approximately 22,440 women are diagnosed with ovarian cancer and 14,080 die of the disease (2). High-grade serous ovarian carcinoma is the most common ovarian cancer subtype and accounts for the majority of these deaths (3). The poor survival rate of ovarian cancer can often be ascribed to a late diagnosis due to the cancer's asymptomatic nature in early stages, a high rate of relapse following first-line treatments, and lack of effective therapies for recurrent, often chemoresistant, cancer (4–6). Second-line therapies that reduce and treat cancer recurrence are needed.

Natural killer (NK) immune cells have garnered attention as a cancer immunotherapeutic due to their ability to kill malignant cells and spare healthy cells (7). NK cell cytotoxicity is regulated through a balance of activating and inhibitory receptors: through the integration of activation and inhibitory signals, NK cells hone their cytotoxic activity against malignant cells without harming healthy cells (7). This attribute prevents off-tumor toxicity. The safety of adoptive NK cell transfer has been demonstrated in the clinical setting (8). Furthermore, the adoptive transfer of NK cells has demonstrated clinical effectiveness in treating hematologic malignancies, underscoring the value of NK cell cancer immunotherapy (8, 9).

Despite the therapeutic potential, NK cell therapies have been of limited value against solid tumors due to an inability to maintain NK cell cytotoxicity in the immunosuppressive tumor environment. Although NK cells are cytotoxic against ovarian cancer cells in vitro through engagement of activating receptors with respective ligands on ovarian cancer cells (10), the tumor microenvironment in vivo, including ascites in the case of ovarian cancer, inhibits NK cell antitumor functions (11–16). In fact, tumor-associated ascites-NK cells are hyporesponsive to tumor targets due to reduced expression of activation receptors, including NKG2D and DNAM-1, driven by chronic engagement with activating receptor ligands expressed or shed by tumor cells (17, 18). In addition, ascites-NK cells overexpress certain
inhibitory receptors, including the immune checkpoint receptor PD-1 that hampers the NK cell antitumor response (19). In the clinical setting, IL2-activated allogeneic NK cells had limited effects in patients with breast and ovarian cancer (20). Furthermore, the adoptive transfer of in vitro–activated autologous NK cells to patients with melanoma or renal cell carcinoma failed to achieve a clinical response as the cytotoxic function of NK cells was impaired (21). If the impairment in function of adoptively transferred NK cells by solid tumors could be overcome, NK cell therapy might be as successful for solid tumors as it already is for hematological malignancies.

NK cells fall into two major subsets: CD56dimCD16+ cytotoxic NK cells and CD56brightCD16− immunoregulatory and poorly cytotoxic NK cells (22). However, preactivation of peripheral blood (PB)-NK cells with IL15 causes CD56bright NK cell antitumor functions to exceed those of CD56dim NK cells. Thus, modulation of CD56bright NK cells can improve their antitumor functions and merit further exploration as a cancer immunotherapy (23).

A feeder cell-based NK cell expansion protocol has been developed that generates NK cells that are clinically effective against leukemia (8). Expanded NK cells may be promising for the treatment of solid malignancies: one study demonstrated that expanded PB-NK cells from healthy donors reduced tumor burden in a cell-line xenograft ovarian cancer model, suggesting expansion may improve NK cell function against solid tumors (24). Our group has expanded NK cells from the PB of breast and ovarian cancer patients and ascites of ovarian cancer patients (25, 26). We found that not only did expansion convert cancer patient NK cells to an activated subset with in vitro cytotoxicity comparable with that of expanded healthy donor NK cells, but that expansion converted immunoregulatory ascsite-NK cells to a subset with in vitro cytotoxicity against ovarian cancer cells. Together, these previous findings indicate the potential for adoptively transferring patients’ own expanded NK cells as an autologous cell therapy for ovarian cancer and other solid malignancies.

In the present study, we show that expansion induces a CD56dimCD16− NK cell population that possesses better in vitro antitumor functions against ovarian cancer cells than IL2-activated unexpanded NK cells, supporting the notion that CD56dim NK cells have untapped cytotoxic potential. Given this enhanced in vitro function, we evaluated the ability of expanded ovarian cancer patient PB- and ascsite-NK cells to reduce tumor burden in vivo using both representative cell-line and autologous patient-derived xenograft models of aggressive human ovarian cancer. We report that expanded ovarian cancer patient PB- and ascsite-NK cells reduced the burden of well-established tumors, enhanced survival, and were effective against a patient’s own (autologous) aggressive and resistant primary ovarian cancer.

Materials and Methods

Ethics statement
Research using human samples was approved by the Hamilton Integrated Research Ethics Board. PB and ascites from high-grade serous ovarian cancer patients with recurrent cancer and PB from healthy donors were obtained with written informed consent. NOD-Rag1null IL2rgnull (NRG) mice were purchased from The Jackson Laboratory and bred in pathogen-free conditions in the Central Animal Facility at McMaster University. All breeding and experiments involving mice were approved by the Animal Research Ethics Board at McMaster University.

Cell culture and in vitro assays
NK cells were expanded from PB mononuclear cells (PBMCs) or ascites cells using IL2 and irradiated K562-feeder cells engineered to express membrane-bound IL21 (K562-mb-IL21), as previously described (25, 27, 28). K562-mb-IL21 cells were kindly provided by Dr. Dean A. Lee (Department of Pediatrics, Nationwide Children’s Hospital, Ohio State University Comprehensive Cancer Center, Columbus, OH, USA) in 2012. K562-mb-IL21 cells were kept in culture for a maximum of 2 months and were not authenticated in the past year. Expanding NK cell cultures were replenished with 100 U/mL IL2 three times per week and irradiated K562-mb-IL21 cells once per week at a 2:1 ratio to NK cells. NK cell were expanded for 3 weeks prior to use in experiments. Unexpanded NK cells were isolated from PBMCs using an NK Cell Enrichment Kit (STEMCELL Technologies; #19055). For in vitro assays, expanded and unexpanded NK cells (106 cells/mL) were incubated overnight with 100 U/mL IL2 (PeproTech; #200-02). NK cell phenotype and IFNγ were assessed via flow cytometry. Cytotoxicity and degranulation assays against ovarian cancer cells were conducted as previously described (25, 26). OVCA8 ovarian cancer cells were obtained in 2015 as a kind gift from Dr. Karen Mossman (McMaster University) and used for experiments at 10th passage. Mycoplasma testing was performed on K562-mb-IL21 and OVCA8 cells in 2015 using PlasmoTest mycoplasma detection kit (InvivoGen; #rep-pt1) according to the manufacturer’s instructions and cells were used from these tested batches. OVCA8 cells were authenticated by the American Type Culture Collection Cell Line Authentication Service in 2017.

Generation of OVCA8-Luciferase cells
OVCA8 cells were transfected with a lentiviral vector containing a luciferase reporter gene. For the production of a third-generation lentivirus, a pCCL-based transfer plasmid was used, which encodes puromycin resistance and enhanced firefly luciferase in a bidirectional promoter system (under control of the minimal cytomegalovirus and human EF-1α promoters, respectively; ref. 29). Self-inactivating, nonreplicative lentivirus was produced using a third-generation system that has been previously described (30, 31). Transduced OVCA8-Luciferase cells were selected for via puromycin selection.

Establishment of patient-derived xenograft ovarian cancer model
Ovarian cancer ascites cells from 5 patients were injected intraperitoneally into NRG mice in PBS or the indicated volume of ascites fluid. Mice were followed for ascites development and survival. Ascites cells were collected at endpoint, defined by body weight plateau or increase with poor body condition (including decrease or cessation of food or water intake due to abdominal distension) compared with control NRG mice. These passaged ascites cells were injected in PBS intraperitoneally into NRG mice at a range of doses (0.25–3 × 106 cells/mouse) to determine optimal dose for consistent engraftment and mice were followed for ascites development and survival. At endpoint, ascites was collected and solid peritoneal tumors were harvested, fixed in 2% paraformaldehyde for 48 hours, embedded in paraffin, cross-sectioned, and stained with H&E. Tumor sections were imaged with a Leica Microscope. CA-125 was quantified using a CA-125
Adoptive transfer of expanded NK cells

OVCAR8-Luciferase or primary ovarian cancer cells (passaged once) were injected intraperitoneally into NRG mice (2.5 x 10^5 cells/mouse) at day 0. At indicated time points after tumor cell injection, 20 x 10^6 expanded NK cells were injected intraperitoneally. A total of 2 x 10^4 IU of IL2 (Promega), a dose that was previously determined to support expanded NK cell survival in vivo (25), was injected intraperitoneally 3/week to support NK cell survival in the absence of a complete host immune system. Control mice received tumor cells and IL2. For experiments with OVCAR8-Luciferase xenografts, tumor burden was quantified 14 minutes after intraperitoneal injection of Luciferin (Perkin Elmer #30356214) via bioluminescence (radiance units: photons/sec/cm^2/sr) using an IVIS Spectrum Imaging System and analyzed using Living Image software (Perkin Elmer). Tumor engraftment was confirmed and bioluminescence was averaged across groups prior to the first NK cell injection. For tumor burden assessment in patient-derived xenografts, abdominal distension was assessed by measuring mouse abdominal circumference as previously described (32). Circumference was measured in line with the iliac crest to ensure consistent measurement across mice. Peritoneal tumors were compared across groups when the first mice reached endpoint. Endpoint across experiments was defined by body weight plateau or increase with poor body condition (including decrease or cessation of food or water intake due to abdominal distension) compared with control NRG mice.

Flow-cytometric staining

Cells were stained with viability dye (eBioscience; #65-0865-14) for 30 minutes. Cells extracted from mice were Fc-blocked for 20 minutes. Extracellular and intracellular staining was conducted as previously described (28). NK cells were gated as live human CD45^-CD56^-CD3^- cells. The following antibodies were used. From BD Biosciences: anti-human Nkp46 (#320906), ULBP1 (#312712), CD112 (#337410), CD155 (#337610), HLA A,B,C (#311406), CD158b (#312606), NKG2A (#562364), CD16 (#302026), NKp30 (#325210), NKp44 (#325108). From R&D Systems: ULBP3/4 (#FAB1380A). ULBP3/4 (#FAB1517a), B7H6 (#FAB1298P). Data acquisition was conducted on a BD LSFortessa.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software. Graphs comparing two conditions were analyzed via unpaired t test. Graphs comparing more than two conditions were analyzed via one-way ANOVA followed by Tukey correction for multiple comparisons. Graphs with two independent variables were analyzed via two-way ANOVA followed by Tukey correction. Survival was analyzed using the log-rank (Mantel–Cox) test followed by Bonferroni correction for multiple comparisons.

Results

Expanded CD56^{superbright}CD16^- NK cells showed enhanced in vitro antitumor function

IL2-activated NK cells have previously failed to induce clinical responses in patients with solid malignancies (20). With the development of NK cell expansion protocols, clinically relevant numbers of K562-mb-IL21-expanded NK cells can be generated and have shown therapeutic antitumor capabilities in patients with hematologic malignancies (8). We sought to determine whether expanded NK cells may have improved function against a solid malignancy. We first compared the in vitro antitumor function of K562-mb-IL21-expanded and unexpanded IL2-activated healthy donor PB-NK cells. Expanded NK cells had enhanced IFNγ expression compared with unexpanded IL2-activated NK cells (Fig. 1A) and superior cytotoxicity against OVCAR8 cells (Fig. 1B). These improved antitumor functions compared with IL2-activated NK cells suggest that expanded NK cells comprise a more functional subset.

CD56^{bright}CD16^- IL15-activated NK cells harbor greater antitumor capacity than CD56^{dim}CD16^- NK cells (23). We have previously reported that expanded NK cells are primarily comprised of a CD56^{bright}CD16^- population (26). Although the phenotype of unexpanded ovarian cancer patient PB- and ascites-NK cells and healthy donor NK cells differed prior to expansion, after expansion these cell populations exhibited similar phenotype and in vitro antitumor functions (26). As we found enhanced in vitro function of expanded NK cells compared with unexpanded NK cells, we further characterized expanded NK cell phenotype and function with respect to intensity of CD56 expression. We found that expanded NK cells are comprised primarily of a CD56^{superbright} population: CD56 brightness in expanded NK cells was greater than that of the CD56^{bright} population in unexpanded IL2-activated NK cells (Fig. 1C). Correspondingly, expanded NK cell CD56 mean fluorescence intensity (MFI) was significantly greater than IL2-activated NK cells due to an increase in both percent CD56^{bright} cells and CD56 expression intensity on a per cell basis (Fig. 1C and D). Although CD56^{bright} PB-NK cells do not express CD16, the majority of the expanded CD56^{bright} NK cell population expressed CD16 (Fig. 1E), in accordance with previous findings (26). Indicative that CD56 expression intensity corresponds with activation state, expanded NK cells had increased CD16 expression with increasing CD56 brightness (Fig. 1F; Supplementary Fig. S1A). The expression of other activation receptors, including CD69, NKG2D, NKp30, NKp44, and NKp46, also increased with CD56 brightness (Fig. 1G and H). In contrast, expression of NKG2A and KIR (CD158a, CD158b, and CD158e1) inhibitory receptors did not significantly change with CD56 brightness (Fig. 1I; Supplementary Fig. S1G–S1I).

We then assessed the antitumor functions of expanded NK cells with respect to CD56 expression intensity in order to determine whether CD56 brightness corresponds with enhanced function. Expanded NK cells were stratified into CD56^{low}, CD56^{bright}, and CD56^{superbright} populations, as gated in Fig. 1C. Indeed, both the percentage of cells expressing IFNγ and the IFNγ MFI increased with CD56 brightness (Fig. 2A–C). MFI was assessed on CD56^{dim}, CD56^{bright}, and CD56^{superbright} NK cell populations in order to assess both numbers of IFNγ^- cells and IFNγ expression intensity on a per-cell basis. Furthermore, NK cell
Figure 1. *Ex vivo* K562-mb-IL21–expanded NK cells are a CD56\textsuperscript{superbright}CD16\textsuperscript{+}–activated subset with greater antitumor functions compared with IL2-activated NK cells. Expanded and unexpanded NK cells were activated overnight with IL2 (100 U/mL). 

A, Percentage of IFN\textgamma expression was compared (n = 6 donors per group).

B, NK cell percent specific lysis of OVCAR8 target cells following 5-hour incubation at a 5:1 effector-to-target ratio (unexpanded group n = 5; expanded group n = 6).

C, Representative flow plots and (D) MFI of CD56 expression (n = 6 donors per group).

E, Proportion of CD56\textsuperscript{bright}CD16\textsuperscript{+} cells of total NK cell populations (n = 5 donors per group). F–O, Expanded NK cell population was stratified by flow cytometry analysis based on CD56 expression. Percent expression of activation receptors (F) CD16 (n = 5 donors), (G) CD69, (H) NKG2D, (I) Nkp30, (J) Nkp44, (K) Nkp46, and inhibitory receptors (L) NKG2A, (M) CD158a, (N) CD158b, and (O) CD158e1 was compared (G–O: n = 3 donors). A–E, Results from two independent experiments. G–O, Results from one experiment. A–E were analyzed via unpaired t test. F–O were analyzed via matched one-way ANOVA. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
degranulation in response to OVCAR8 tumor targets increased with increasing CD56 brightness (Fig. 2D and E). Thus, K562-mb-IL21-expansion produces a CD56superbright/CD16+ -activated NK cell population with enhanced antitumor functions compared with unexpanded IL2-activated NK cells. Activation state and antitumor functions of these cells increased with increasing CD56 expression.

Expanded ovarian cancer patient NK cells effective in a xenograft ovarian cancer model

Our group has reported that K562-mb-IL21-expansion converts initially impaired cancer patient PB- and tumor-associated NK cells to cytotoxic NK cells with in vitro antitumor functions comparable with those of expanded healthy donor NK cells (25, 26). Here, we asked whether expanded cancer patient NK cells could reduce tumor burden in vivo against an established solid tumor.

We compared the therapeutic capacity of expanded ovarian cancer patient PB-NK cells (OCP PB-NK), ascites-NK cells (OCP ascites-NK), and healthy donor PB-NK cells (HD PB-NK) to reduce tumor burden in a murine ovarian cancer xenograft model generated using OVCAR8-Luciferase cells. Expanded OCP PB-NK, OCP ascites-NK, and HD PB-NK cells were adoptively transferred intraperitoneally to mice at indicated time points, for a total of 5 NK cell injections (Fig. 3A). NK cell treatments began 2 days following injection of OVCAR8-Luciferase cells as this was determined to be the time point by which tumor cells had consistently engrafted and bioluminescence was similar across mice. Five NK cell injections were administered in order to determine whether multiple injections of expanded NK cells (i) could be well tolerated and (ii) could exert a therapeutic effect in this aggressive model. Tumor burden was quantified at regular intervals until bioluminescence became saturated in the tumor-only group. The adoptive

Figure 2.

Antitumor functions of expanded NK cells increase with increasing CD56 brightness. Expanded NK cell population was stratified by flow cytometry analysis based on CD56 expression intensity. A, Percent (n = 6 donors), B, MFI (n = 6 donors), and C, representative flow plots of IFNγ expression. D, Expanded NK cell percent CD107a expression following 5-hour incubation with OVCAR8 target cells (n = 5 donors). E, Representative flow plots of CD107a expression. Results are from two independent experiments and were analyzed via matched one-way ANOVA; *, P < 0.05; **, P < 0.01.
transfer of expanded NK cells reduced detectable tumor burden to levels equivalent to control mice with no tumor (Fig. 3B and C). Expanded NK cell groups from all sources demonstrated comparable reduction in tumor burden.

In addition to eliminating macroscopic tumor burden, the five NK cell treatments enhanced median survival time 3.4 to 5.2 times over survival time of tumor-only controls (Fig. 3D). NK-treated mice experienced tumor regrowth at variable time points after cessation of NK cell treatment (a range of 45–135 days following last NK cell treatment) and were sacrificed when they reached the same disease endpoint criteria as untreated tumor-only control mice. No significant difference in survival was observed between HD PB-NK and OCP ascites-NK. There was a nonsignificant trend for improved survival of HD PB-NK versus OCP PB-NK. However, OCP ascites-NK cells significantly improved survival as compared with OCP PB-NK cells. These results indicate that not only are expanded NK cells effective in reducing tumor burden against solid tumors, but expanded OCP ascites-NK cells are as effective as expanded HD PB-NK cells, indicating the potential for autologous NK cell therapy.

Expanded OCP-NK cells reduced burden of established ovarian cancer in xenograft mice

The immunosuppressive environment produced by established solid tumors reduces effectiveness of NK cell therapy. We therefore asked whether expanded OCP-NK cells could maintain the therapeutic effect observed in Fig. 3 if treatment began at a later time point. This allowed a tumor environment to more fully establish following engraftment. We injected expanded OCP PB-NK cells at days 8 and 10 following OVCAR8-Luciferase injection, at approximately one third of the median survival time for untreated mice (Fig. 4A). Mice were monitored until the bioluminescent signal became saturated in the control group. We found that only two injections of expanded OCP-NK cells reduced macroscopic tumor burden to undetectable levels (comparable with mice with no tumor; Fig. 4B and C). Thus, expanded OCP-NK cells show antitumor activity against established ovarian cancer.

**Translational patient-derived xenograft model of primary human ovarian cancer**

Our findings that expanded OCP PB-NK and ascites-NK cells are effective at reducing tumor burden in an ovarian cancer cell-line xenograft model indicate potential for autologous NK cell therapy. We therefore asked whether expanded OCP-NK cells can exert a therapeutic effect against the patient’s own primary ovarian cancer. To address this question, we first established a translational patient-derived xenograft ovarian cancer murine model. We injected unpassaged ovarian cancer patient ascites cells in PBS or ascites fluid intraperitoneally into NRG mice and monitored ascites development and tumor burden (Fig. 5A). Injection with ascites fluid supported the development and progression of ovarian cancer and reduced the number of cells needed for engraftment (Fig. 5B). However, the volume of ascites fluid was critical: we found that 1 to 2 mL ascites fluid supported engraftment without inducing lethal toxicities (Fig. 5C). Upon subsequent passage, as few as $2.5 \times 10^7$ passaged cells per mouse were needed for consistent engraftment. Ascites fluid was not required for consistent engraftment in second passage. The progression of ovarian cancer in these patient-derived xenograft murine models paralleled clinical progression: mice expressed the clinical ovarian cancer biomarker CA-125 (Fig. 5D) and developed ascites and solid tumors in the peritoneal cavity that retained epithelial characteristics (Fig. 5E–G).
Expanded OCP-NK cells retained a cytotoxic phenotype in autologous tumors

Establishment of the patient-derived xenograft mice enabled us to assess the adoptive transfer of expanded OCP-NK cells in an autologous model of patients’ own cancer. We asked whether the cytotoxic phenotype of expanded NK cells was affected by established ovarian cancer, and whether expanded NK cells from different sources (autologous OCP or allogeneic HD; PB- or ascites-derived) were differentially affected. We adoptively transferred expanded autologous OCP PB-NK, OCP ascites-NK, or allogeneic HD PB-NK cells intraperitoneally into mice with either no tumor (controls) or patient-derived xenograft mice with visible ascites. Peritoneal fluid was collected from the mice 48 hours following adoptive NK cell transfer. NK cells were identified as live human CD45+CD56+CD3-/C0 cells (Fig. 6A). Expanded NK cells in all groups demonstrated an enriched CD56superbright population in ascites, compared with in mice without tumors (Fig. 6B). However, in contrast to the ascites-induced CD56brightCD16− immunoregulatory phenotype of unexpanded NK cells (12), expanded NK cells maintained high expression of CD16 (Fig. 6C) and predominantly consisted of a CD56superbrightCD16+ activated population. In addition, expanded NK cells increased or maintained expression of activation receptors Nkp46 and NKP30 in ascites (Fig. 6D and E). These results indicate that expanded NK cells from all sources maintain an activated CD56superbrightCD16+ cytotoxic phenotype with enhanced or sustained expression of activation receptors at the tumor site. In contrast, with a lack of activation signals in nontumor-bearing mice, expanded NK cells revert to a less activated CD56dim subset.

Expanded ovarian cancer patient NK cells reduce burden of autologous ovarian cancer

Given the maintenance of a cytotoxic phenotype in an autologous tumor model, we next investigated the therapeutic capacity of expanded OCP PB-NK and ascites-NK cells to reduce tumor burden against autologous ovarian cancer. We asked whether expanded OCP-NK cells could exert a therapeutic effect against autologous ovarian cancer in even a resistant and aggressive cancer model that constitutively expressed MHCI. We have previously reported that ovarian cancer cells from different patients have variable susceptibility to NK cell killing in vitro (26). For assessing the adoptive transfer of expanded NK cells in an autologous model, we used primary ovarian cancer cells that we had identified as resistant to NK cell killing in vitro (26). These ovarian cancer cells remained resistant to NK cell killing following passaging in mice and more susceptible cells retained susceptibility (Fig. 7A). Because inhibition by self-MHC is an impediment to autologous NK cell therapy, we verified that the ovarian cancer cells used expressed MHCI
Furthermore, primary ovarian cancer cells have variable in vivo aggressiveness, with mice reaching endpoint in a range of 20 to 150 days following tumor cell injection. Cells used in the current experiment were aggressive, with untreated mice reaching endpoint within 24 days (Fig. 7C).

Expanded OCP PB-NK, OCP ascites-NK, or HD PB-NK cells were adoptively transferred intraperitoneally at indicated time points after injection of primary ovarian cancer cells in PBS or ascites fluid. Because survival time of untreated xenografts in this model is similar to that of OVCAR8-Luciferase xenografts in Fig. 3, expanded NK cell treatment began at day 2 following tumor cell injection to allow similar time for tumor cell engraftment. Adoptive transfer of expanded NK cells delayed the onset and progression of ascites compared with control mice, as measured by abdominal circumference (Fig. 7D). Furthermore, no significant difference in abdominal circumference was observed among any of the NK cell groups. Treatment with expanded NK cells precluded development of large peritoneal tumors, which did develop in control mice (Fig. 7E).

Our results demonstrate not only that expanded NK cells are effective against primary ovarian cancer, but also that expanded OCP PB-NK and ascites-NK cells have a therapeutic effect against aggressive and resistant autologous ovarian cancer.
Discussion

To date, the adoptive transfer of NK cells has been ineffective at treating solid malignancies because NK cell activation and effector function are not maintained in the solid tumor environment. However, we report that cytotoxic expanded CD56\textsuperscript{superbright} CD16\textsuperscript{+} NK cells may bypass this limitation. We demonstrate that expanded cancer patient NK cells can be therapeutically beneficial against an established solid malignancy. We report that expanded OCP PB- and ascites-NK cells reduce tumor burden and improve survival in allogeneic and autologous tumor settings using translational human ovarian cancer models.

CD56\textsuperscript{dim} NK cells are usually cytotoxic, whereas CD56\textsuperscript{bright} NK cells are immunoregulatory and poorly cytotoxic (22). However, preactivation of PB-NK cells with IL15 augments CD56\textsuperscript{bright} NK cell antitumor functions, surpassing those of CD56\textsuperscript{dim} NK cells (23). Although such results indicate potential for CD56\textsuperscript{bright} PB-NK cells as a cancer immunotherapeutic, CD56\textsuperscript{bright} NK cells form only a minor portion of PB-NK cells and are thus not available in numbers relevant for clinical therapies.

Here, we identified an expansion-induced CD56\textsuperscript{superbright} CD16\textsuperscript{+} NK cell population that produces more IFN\gamma and shows greater in vitro cytotoxicity against ovarian cancer cells than IL2-activated NK cells. The antitumor functions of these expanded NK cells increase with CD56 expression intensity, which further contributes to the shifting paradigm that certain populations of CD56\textsuperscript{bright} NK cells harbor greater antitumor potential compared with CD56\textsuperscript{dim} NK cells (23). Expansion is a feasible method for obtaining clinically relevant numbers of cytotoxic CD56\textsuperscript{bright} NK cells because K562-mb-IL21-expansion produces robust numbers of NK cells, the majority of which are CD56\textsuperscript{superbright} CD16\textsuperscript{+}. The function of these CD56\textsuperscript{superbright} CD16\textsuperscript{+} NK cells exceeds the antitumor functions of unexpanded IL2-activated NK cells; these CD56\textsuperscript{superbright} CD16\textsuperscript{+} NK cells will likely be more effective than unexpanded NK cells against solid malignancies.

Although NK cells from different sources (HD PB-NK, OCP PB-NK, and OCP ascites-NK) have different functionality prior to expansion (16), we found that after expansion they all reduce ovarian cancer tumor burden and improve survival following intraperitoneal delivery in a cell-line xenograft ovarian cancer murine model. Expanded OCP-NK cells maintain their therapeutic ability to reduce tumor burden against well-established tumor. The effectiveness of expanded OCP-NK cells demonstrates that K562-mb-IL21-expansion converts even previously impaired cancer patient NK cells to a cytotoxic subset capable of exerting a therapeutic effect \textit{in vivo} against an established solid malignancy.

Although unexpanded ascites-NK cells have immunoregulatory properties and are poorly cytotoxic, our group has previously demonstrated that expanded ascites-NK cells demonstrate in vitro cytotoxicity against ovarian cancer cells similar to that of expanded PB-NK cells (26). The current study extends these findings to \textit{in vivo} setting by demonstrating that after expansion, ascites-NK cells are as able as PB-NK cells to reduce ovarian cancer tumor burden \textit{in vivo} and in fact induced a 5.2 times increase in median survival compared with untreated mice. Thus, immunosuppressed tumor-associated

Figure 6.
Expanded NK cells maintain a cytotoxic phenotype in an autologous ovarian cancer microenvironment. Expanded OCP PB-NK, OCP ascites (Asc)-NK, or HD PB-NK cells were adoptively transferred intraperitoneally to PDX mice with visible signs of ascites or control NRG mice with no tumor. Forty-eight hours following adoptive NK cell transfer, ascites or peritoneal fluid was collected from mice and cells were stained with NK cell markers for flow-cytometric analysis of NK cell phenotype. A, Representative flow plot of gating strategy. NK cells were identified as human hCD45 hCD56 hCD3. Proportion of (B) CD56\textsuperscript{superbright} NK cells, (C) CD16 expression, (D) NKp46 expression, and (E) NKp30 expression on NK cells. Results were analyzed via two-way ANOVA; *, P < 0.05; ***, P < 0.001; ****, P < 0.0001 (tumor group n = 5; no tumor control group n = 3 from one experiment).
NK cells can be modulated through expansion to induce a therapeutic effect in tumor reduction and survival. These results identify tumor-associated NK cells as a potential NK cell source for therapy. Because large volumes of ascites are collected during standard-care paracentesis, ascites offers an efficient source of NK cells without imposing additional procedures on patients.

We established a clinically relevant patient-derived xenograft model of ovarian cancer to study autologous NK cell therapy. Using this model, we demonstrate that expanded OCP PB- and ascites-NK cells are capable of reducing autologous tumor. This therapeutic effect was observed despite the fact that these primary ovarian cancer cells were resistant to NK cell killing in vitro, constitutively expressed autologous MHCI, and were aggressive in vivo. Autologous NK cells have been clinically unsuccessful in reducing tumor burden in the context of solid malignancies and were found to have impaired functionality following adoptive transfer (21). Our findings indicate that the cytotoxic NK cell subset induced by K562-mb-IL21 expansion may be more effective for autologous NK cell therapy. These clinically relevant findings support future clinical assessment of patients’ own expanded NK cells as a therapy for ovarian cancer.

Our assessment of expanded NK cell phenotype following adoptive transfer revealed that expanded NK cells maintain a CD56supertightCD16phenotype with activation receptor expression at the tumor site but lose this activated phenotype when injected into mice with no tumor, where there is a lack of activation signals. This finding indicates that although expanded NK cells can lose their activated state, they remain activated upon interaction with established tumor. NKp30 expression, which recognizes B7-H6 on tumor cells, may be necessary for maintaining expanded NK cell activation, as a previous study demonstrated that NKp30 was downregulated in ascites-NK cells, which impaired NK cell cytototoxicity and IFNγ production (18). Maintenance of CD16 expression by expanded NK cells indicates potential for combining expanded NK cell therapy with a monoclonal antibody targeting a tumor antigen. Monoclonal antibody therapies have had little success against ovarian cancer, largely due to the impaired function and downregulation of CD16 on NK cells.
cells at the tumor site (12, 14, 16, 34). Thus, combining antibody therapy with the adoptive transfer of expanded NK cells that maintain CD16 expression may provide an additional stimulatory signal for expanded NK cells, producing a reciprocally supportive therapeutic combination.

Overall, this study suggests that expanded autologous NK cells are a viable therapy for ovarian cancer that warrants clinical assessment. Overcoming the immunosuppressive challenges posed by solid tumors has been a hurdle for cancer immunotherapies. The present study demonstrates that K562-mbIL21-expanded NK cells may address these hurdles as they show the ability to reduce tumor burden of established tumors in translational models. These results support future clinical investigation to determine whether autologous NK cell therapy can address the longstanding need for an effective second-line therapy to improve the prognosis of ovarian cancer and other solid malignancies.

Disclosure of Potential Conflicts of Interest
JA Hammill has ownership interest in Triumvira Immunologics, Inc. D.A. Lee is Chair of the Medical and Scientific Advisory Board for CytoSen Therapeutics, has received honoraria from speakers bureau honoraria from Miltenyi Biotec, has ownership interest in CytoSen Therapeutics and Zopharm Oncology, Inc., and is a consultant/advisory board member for Courier Therapeutics. H.W. Hirte is a consultant/advisory board member for AstraZeneca and Roche. No potential conflicts of interest were disclosed by the other authors.

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References


Expanded CD56<sup>superbright</sup>CD16<sup>+</sup> NK Cells from Ovarian Cancer Patients Are Cytotoxic against Autologous Tumor in a Patient-Derived Xenograft Murine Model

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