



Cytokine-induced memory-like natural killer cells have enhanced function, proliferation, and *in vivo* expansion against ovarian cancer cells

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HIGHLIGHTS

- Cytokine-induced memory-like (CIML) NK cells demonstrate enhanced function and proliferation against ovarian cancer cells.
- A novel killing assay demonstrated enhanced CIML NK-cell-mediated cytolytic function against ovarian cancer in real time.
- The functional and proliferative ability within immunosuppressive ascites was rescued with healthy donor CIML NK cells.
- Human CIML NK cells exhibit potent antitumor effects within an *in vivo* mouse model of ovarian cancer.

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ABSTRACT

Objective. Natural killer (NK) cells are lymphocytes well suited for adoptive immunotherapy. Attempts with adoptive NK cell immunotherapy against ovarian cancer have proven unsuccessful, with the main limitations including failure to expand and diminished effector function. We investigated if incubation of NK cells with interleukin (IL)-12, IL-15, and IL-18 for 16 h could produce cytokine-induced memory-like (CIML) NK cells capable of enhanced function against ovarian cancer.

Methods. NK cells were preactivated briefly with IL-12, IL-15, and IL-18, rested, then placed against ovarian cancer targets to assess phenotype and function *via* flow cytometry. Real-time NK-cell-mediated tumor-killing was evaluated. Using ascites cells and cell-free ascites fluid, NK cell proliferation and function within the immunosuppressive microenvironment was evaluated *in vitro*. Finally, CIML NK cells were injected intraperitoneal (IP) into an *in vivo* xenogeneic mouse model of ovarian cancer.

Results. CIML NK cells demonstrate enhanced cytokine (IFN- γ) production and NK-cell-mediated killing of ovarian cancer. NK cells treated overnight with cytokines led to robust activation characterized by temporal shedding of CD16, induction of CD25, and enhanced proliferation. CIML NK cells proliferate more with enhanced effector function compared to controls in an immunosuppressive microenvironment. Finally, human CIML NK cells exhibited potent antitumor effects within a xenogeneic mouse model of ovarian cancer.

Conclusions. CIML NK cells have enhanced functionality and persistence against ovarian cancer *in vitro* and *in vivo*, even when exposed to ascites fluid. These findings provide a strategy for NK cell-based immunotherapy to circumvent the immunosuppressive nature of ovarian cancer.

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1. Introduction

Traditionally seen as part of the innate immune response, natural killer (NK) cells are lymphocytes well suited for adoptive immunotherapy

due to their ability to kill target cells without prior sensitization or restriction of major histocompatibility complex (MHC) molecule expression [1,2]. NK cells comprise about 5–10% of circulating lymphocytes and are phenotypically defined by CD56⁺CD3⁻ surface expression by flow cytometry [3,4]. The activity of NK cells is highly regulated by a variety of germline-encoded inhibitory and activating receptors [5]. NK cells can be divided into CD56^{bright}CD16⁻ or CD56^{dim}CD16⁺ populations. Developmentally immature CD56^{bright}CD16⁻ NK cells are capable of producing abundant cytokines, particularly interferon gamma (IFN- γ), immediately

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after activation but possess little direct cytolytic functions [6]; while mature CD56^{dim}CD16⁺ NK cells can kill transformed cells *via* perforin/granzyme release or death receptor (Fas or TRAIL) pathways [7,8].

Epithelial ovarian cancer (EOC) is a rare but lethal malignancy, with an estimated 14,070 deaths expected in the United States for 2018 [9]. Despite aggressive treatment with surgery and systemic chemotherapy, up to 80% will relapse and eventually die, highlighting the pressing need for exploratory and novel therapeutic strategies [10]. Compelling evidence over the past decade indicates ovarian cancer as an immunogenic tumor [11–13]. However, numerous studies document limited NK cell infiltration within the primary ovarian tumor and poor NK cell expansion within the abdominal cavity [11,14–16]. In addition, the tumor microenvironment appears to be immunosuppressive resulting in impaired proliferation and function of NK cells [17–20]. Our group has recently shown the IL-15 superagonist complex (ALT-803) can be used to expand ovarian cancer patient NK cells and induce ovarian cancer control in a xenogeneic mouse model [21]. Additional methods, including short-term incubation with cytokine cocktails may further enhance NK cell effector function to overcome the immunosuppressive environment encountered in ovarian cancer.

Cooper et al. demonstrated short-term cytokine activation of murine NK cells with Interleukin (IL)-12, IL-15, and IL-18 resulted in durable and enhanced IFN- γ production upon re-stimulation weeks later [22]. These NK cells expanded and passed the enhanced functionality on to daughter cells, thus were named cytokine-induced memory-like (CIML) NK cells. Ni et al. adoptively transferred murine CIML NK cells into lymphoma or melanoma-bearing irradiated mice with a profound antitumor effect [23]. Similar findings have been described for cytokine-induced human NK cells *in vitro* [24]. Furthermore, human CIML NK cells demonstrated enhanced tumor control and survival in *in vivo* xenogeneic hematologic and melanoma mouse models [25,26]. Recently, the first-in-human phase I clinical trial reported adoptive transfer of memory-like NK cells and demonstrated clinical response in 5 of 9 patients with acute myeloid leukemia, including 4 complete remissions [27]. In the present study, we investigated if short-term preactivation of human NK cells with IL-12, IL-15, and IL-18 enhances *in vitro* and *in vivo* function against ovarian cancer compared to naïve NK cells.

2. Methods

2.1. Sample processing and cell culture

Blood from healthy donors was obtained after receipt of written informed consent at Memorial Blood Bank (Minneapolis, MN). Use of peripheral blood mononuclear cells (PBMCs) from donors was approved by the Committee on the use of Human Subjects in Research at the University of Minnesota. Donor PBMCs were isolated by Ficoll-Paque (GE Healthcare) centrifugation. Fresh cells were used unless otherwise noted. Frozen specimens used in assays were isolated in the same manner and then cryopreserved in 10% DMSO/90% FBS. PBMCs were plated at $3\text{--}5 \times 10^5$ cells/well in 96 well round bottom plates and preactivated for 16 h using [rhIL-12 (10 ng/mL) + rhIL-15 (1 ng/mL) + rhIL-18 (50 ng/mL)] or low-dose control rhIL-15 (1 ng/mL) conditions in RPMI-10 medium with 10% FBS as previously described [24]. Following preactivation, cells were washed and cultured in RPMI-10 supplemented with rhIL-15 (1 ng/mL) to support survival. Fresh media was replaced on day 7 for longer assays.

NK cells for the IncuCyte® assays were purified from fresh healthy donor PBMCs using an enrichment kit with magnetic beads (StemCell Technologies, $\geq 90\%$ CD56⁺ CD3⁻). For the *in vivo* xenogeneic mouse experiment, PBMCs were CD3/CD19 depleted with magnetic beads (StemCell Technologies, $\geq 95\%$ CD3⁻ CD19⁻). After enrichment, NK cells were preactivated with IL-12, IL-15, and IL-18 or control IL-15 as described above.

The University of Minnesota Cancer Center Tissue Procurement Facility obtained ascites samples from 10 patients diagnosed with ovarian cancer following approval from the Institutional Review Board. All specimens were collected in women diagnosed with advanced-stage high-grade serous ovarian or primary peritoneal carcinoma at time of primary debulking surgery. Cells were pelleted, lysed for red blood cells, cryopreserved in 10% DMSO/90% FBS and stored in liquid nitrogen. The ascites supernatant from these patients was stored at -80°C . Ovarian cancer cell lines (MA148, A1847, OVCAR5, SKOV3) were maintained as previously described [28].

2.2. Reagents

The following anti-human antibodies were used: CD56 PE-Cy7 (HCD56), CD16 BV711 (3G8), CD25 BV650 (BC96), CD69 FITC (FN50), CD178 PE (NOK-1), CD95 BV421 (DX2), HLA-DR APC-Cy7 (L243), CD107a FITC (LAMP-1; H4A3), IFN- γ BV421 (4S.B3), TNF- α AF647 (MAb11), and CD45 BV605 (HI30; all BioLegend); CD3 PE-CF594 (UCHT1), CD57 BV605 (NK-1), and CD25 FITC (M-A251; all BD Biosciences); and NKG2A APC (Z199; Beckman Coulter). The following endotoxin-free recombinant human (rh) cytokines were used: rhIL-12 and rhIL-18 (R&D Systems); and rhIL-15 (NCI). ALT-803 was obtained from Altor Bioscience (NANT company, Miramar, FL).

2.3. Functional and proliferation assays

Healthy donor PBMCs or unselected ascites cells from ovarian cancer patients were activated for 16 h (IL-12/15/18 or IL-15 alone) and harvested after 7 or 14 days of *in vitro* rest, counted, and co-cultured with K562 leukemia targets or ovarian cancer cell lines (MA148, A1847, OVCAR5, SKOV3) at an effector to target (E:T) ratio of 1:1 for 4 h in a 96-well round-bottom plate. NK cell degranulation, *via* CD107a staining, and cytokine production, *via* IFN- γ and TNF- α staining, was carried out as previously described [21]. For proliferation experiments, cells were labeled with CellTrace Violet Cell Proliferation Dye (ThermoFisher Scientific) per manufacturer's protocol and washed prior to preactivation with IL-12, IL-15, or IL-18 or control IL-15 conditions for 16 h. Cells were washed and then incubated for 7 days prior to harvest.

For ascites supernatant experiments, healthy donor PBMCs were preactivated as above and then placed in 50% solution of cell free ascites fluid from 5 different patients with ovarian cancer. After 7 days, cells were harvested, counted, and functional assays performed as above with a 2:1 E:T ratio.

2.4. NK-cell-mediated tumor-killing assay

The NK-cell-mediated tumor-killing assay was performed based on manufacturer's protocol (Essen Bioscience). Enriched NK cells were preactivated as described above or control conditions (IL-15 alone) for 16 h, washed to remove cytokines, and then rested in low-dose IL-15 for 7 days prior to use in the study. NK cells from both groups were co-cultured at a 2:1 E:T ratio with nuclear-restricted red fluorescent protein (RFP)-expressing SKOV3 ovarian tumor cells for 84 h. The addition of caspase 3/7 reagent (Essen Bioscience) allowed for detection of apoptosis on SKOV3 cells. Each condition was carried out in two independent experiments. Cells were imaged every 30 min. Green-fluorescent cells were counted as dead cells. Normalized killing of tumor cells calculated by:

$$\frac{\bar{x}(\text{SKOV3 cells in treatment} \times \text{at time point } y) - \bar{x}(\text{Overlap count in treatment} \times \text{at time point } y)}{\bar{x}(\text{SKOV3 cells in no treatment at time point } y) - \bar{x}(\text{Overlap count in no treatment at time point } y)}$$

2.5. In vivo studies

NOD/SCID/ γ c $^{-/-}$ (NSG) mice (Jackson Laboratories) were maintained and procedures conducted under the guidelines approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Mice (8–12 week old female) were injected intraperitoneally (IP) with 2×10^5 luciferase-expressing MA148 ovarian cancer cells and sub-lethally irradiated (225 cGy) 3 days later to allow for maximal NK cell engraftment [29]. On day 1 post-radiation, healthy human donor NK cells (1×10^6 cells in 200 μ L PBS) preactivated with IL-12, IL-15, and IL-18 or control IL-15 conditions as described above from a CD3/CD19 depleted product were injected IP. All mice received ALT-803 (1.25 μ g/injection) subcutaneous injections and were imaged for bioluminescence on days 6, 13, and 20 using the Xenogen IVIS imaging system (Caliper Life Science, Hopkinton, MA). On day 25, mice were euthanized and a postmortem peritoneal lavage performed to obtain NK cells for phenotype and functional assays.

2.6. Flow cytometry analysis

Cells were stained and analyzed using LSRII (BD Biosciences) cytometer as described previously [30]. Data was analyzed with FlowJo Version 10.2 software and plotted using GraphPad Prism Version 7.0c software.

2.7. Statistical analysis

Student *t*-tests (GraphPad Prism Version 7.0c) were used to compare means unless otherwise noted in figure legends. Grouped data represented as mean \pm standard error of the mean (SEM). Multiple comparisons were analyzed with ANOVA tests listed in figure legends. *P* < .05 was considered statistically significant.

3. Results

3.1. CIML NK cells demonstrate enhanced IFN- γ and TNF- α production against ovarian cancer target cells

It was previously established that human NK cells preactivated with IL-12, IL-15, and IL-18 demonstrate a memory-like response against leukemia target cells [24]. To evaluate the response against ovarian cancer targets, human PBMCs were preactivated with CIML conditions (IL-12, IL-15, and IL-18) or control conditions (IL-15 alone) for 16 h, washed to remove cytokines, then placed in low-dose IL-15 for a prolonged rest period (Fig. 1A). After 7 days of rest, CIML NK cells from PBMCs displayed enhanced IFN- γ and TNF- α production against K562 leukemia control cells and MA148, A1847, OVCAR5, and SKOV3 ovarian cancer cells (Fig. 1B and C). No significant difference was seen in CD107a (LAMP-1) surface expression, used as a surrogate for cytotoxicity between CIML NK cells and control NK cells from the PBMCs of the same donors (Fig. 1D). To demonstrate specificity for tumor cells and lack of toxicity towards normal autologous cells, we performed the same experiment with both PBMCs and enriched NK cells against self (autologous) B and T cells (Supplemental Fig. S1A–F). The CIML NK cells did not show appreciable activity when cultured against self B and T cells. These findings demonstrate preactivated human NK cells exhibit extended cytokine production against ovarian cancer cell targets and are safe against autologous or normal cells.

3.2. CIML NK cells demonstrate enhanced killing of SKOV3 ovarian tumor cells in real-time

Although the NK cell CD107a (degranulation) data seemed to indicate CIML NK cells do not induce enhanced cytotoxicity in a short-term assay against ovarian cancer tumor targets, their impact as direct

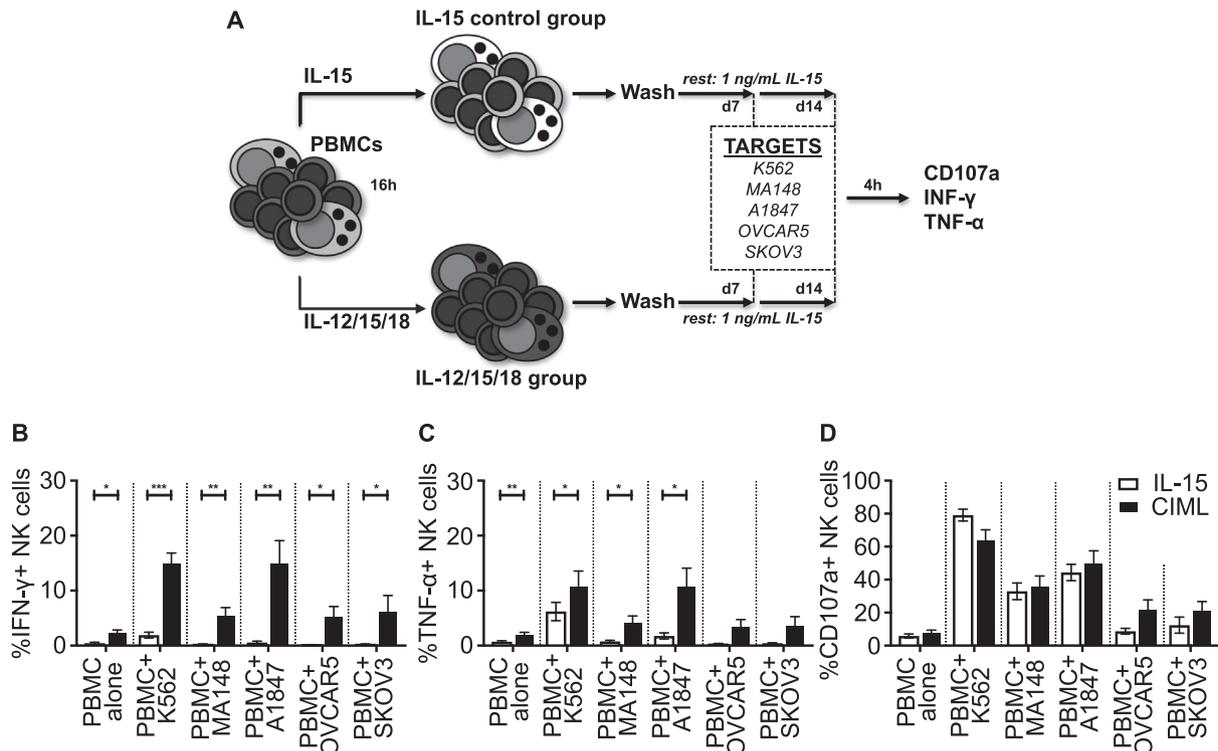


Fig. 1. Cytokine activation with IL-12, IL-15, and IL-18 results in NK cells with enhanced IFN- γ and TNF- α production against ovarian cancer targets. (A) Schema of experimental design with IL-15 control group and IL-12/15/18 group. Freshly isolated PBMCs from healthy donors were incubated with IL-15 (1 ng/mL) or IL-12 (10 ng/mL) + IL-15 (1 ng/mL) + IL-18 (50 ng/mL) for 16 h, washed to remove cytokines, then placed with 1 ng/mL of IL-15 to maintain survival for 7 or 14 days. At time of functional assay, effectors were placed with K562 or ovarian tumor targets at a 1:1 effector (PBMC) to target (tumor) ratio for 4 h. Summary data shown as means \pm SEM percentage of (B) IFN- γ , (C) TNF- α and (D) CD107a NK cells (gated on live, CD56 $^{+}$ CD3 $^{-}$) by flow cytometry (*n* = 8; 4 independent experiments for K562, MA148, and A1847; *n* = 7 for OVCAR5 and *n* = 6, 3 independent experiments for SKOV3). Paired *t*-test (B–D) used to compare statistical differences. **P* < .05; ***P* < .01; ****P* < .001.

ovarian tumor killers in a longer assay was assessed using the IncuCyte® Zoom technology. This platform allows for real-time measurement of cell death over long time periods using automated image analysis software. Imaging was performed every 30 min for direct, real-time measurement of cell death. Representative phase, red fluorescent (nuclear-restricted RFP), and green fluorescent (Caspase 3/7 substrate)-merged images at 12 h illustrate killing of tumor cells with control and CIML NK cells (Fig. 2A). Normalization of the killing of tumor cells at each time point demonstrated enhanced killing with CIML NK cells (Fig. 2B). Cultures with CIML NK cells had a drastic reduction in viable tumor cell count measured by red nuclei/well over time (Fig. 2C and D). Finally, pooled analysis of four donors for the ratio of dead cells to total cells was evaluated at 0, 12, 40, 60, and 80 h (Fig. 2E). These results demonstrate significantly higher rates of killing of SKOV3 ovarian cancer cells with CIML NK cells compared to naïve NK cells following one week of rest *in vitro*.

3.3. Phenotypic changes in healthy donor peripheral blood NKs and NK cells from ascites of patients with ovarian cancer following cytokine activation

We next sought to evaluate the phenotypic alterations induced by preactivation with cytokines on NK cells from healthy donor PBMCs and on NK cells from the ascites (ASC) of patients with ovarian cancer. Preactivation resulted in decreased expression of CD16 on day 1 that recovered and was significantly higher on day 7, both in NK cells from healthy PBMCs and ASC (Fig. 3A and E). As previously reported, cell

surface CD25 expression was induced following cytokine activation on day 1 assay in both NK cells from PBMC and ASC cells (Fig. 3B) [25]. However, expression returned to baseline levels on day 7 after *in vitro* rest and was not statistically different from controls (Fig. 3F). In comparison to controls, preactivated PBMC and ASC cells had increases NK cell surface density of Fas that continued to increase over the course of the week (Fig. 3C and G). Both preactivated PBMC and ASC cells demonstrated a higher proportion of CD69+ NK cells on day 1 and HLA-DR+ NK cells on day 7 (Fig. 3D and H). These data indicate overnight co-treatment with IL-12, IL-15, and IL-18 leads to robust NK cell activation characterized by temporal shedding of CD16 and induction of CD25 with an extended presence of markers involved in cell death.

3.4. CIML NK cell IFN- γ production in healthy PBMC and ASC cells

To confirm the activation marker findings in a functional assay, preactivation with CIML conditions was carried out to evaluate a memory-like IFN- γ response in NK cells from cryopreserved PBMC and ASC cells from patients with ovarian cancer. Day 1 and day 7 functional assays were performed on cryopreserved PBMCs from healthy donors or ascites cells from advanced stage high-grade serous ovarian cancer patients. Compared to the low-dose IL-15 PBMC and ASC cells, CIML PBMC and ASC NK cells demonstrated enhanced cytotoxicity by increased CD107a surface expression against both leukemia and ovarian cancer cells on day 1 (Fig. 4A). The healthy CIML PBMC NK cells against ovarian cancer targets demonstrated similar cytotoxicity as naïve NK

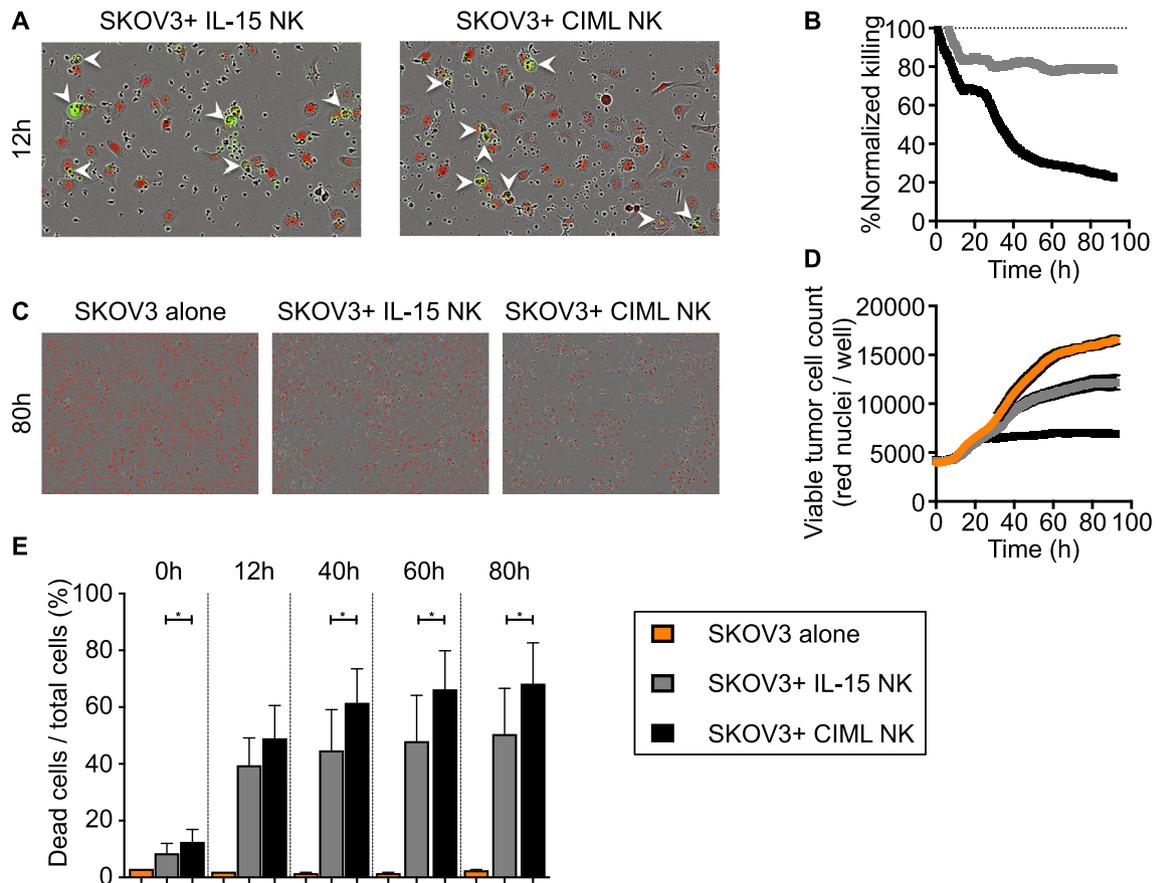


Fig. 2. Cytokine activation of human NK cells with IL-12, IL-15, and IL-18 demonstrates enhanced killing of SKOV3 ovarian tumor cells in real time following 1 week of rest. Freshly isolated PBMCs from healthy donors were enriched for NK cells and then incubated with IL-15 (1 ng/mL) or CIML conditions for 16 h, washed to remove cytokines, then placed with 1 ng/mL of IL-15 to maintain survival. After 7 days, NK cells were then co-cultured with SKOV3 ovarian tumor cells with the addition of Caspase 3/7 substrate for real-time measurement of cell death. (A) Representative phase, red fluorescent (nuclear-restricted RFP), and green fluorescent (Caspase 3/7 substrate) images of SKOV3 co-cultured with NK cells previously treated with IL-15 or CIML conditions at 12 h. Engagement of NK cells with SKOV3 targets highlighted with arrow heads. (B) Representative experiment showing normalized killing of tumor cells over time. (C) Representative phase, red fluorescent (nuclear-restricted RFP) images at 80 h demonstrating viable tumor cell count over time. (D) Representative experiment quantifying viable tumor cell count over time. (E) The quantitative ratio of dead cells to total cells at 0 h, 12 h, 40 h, 60 h, and 80 h. Scale bar, 100 μ m. Each condition (SKOV3 alone, SKOV3+ IL-15 NK, or SKOV3+ CIML NK) carried out in replicates of 6 in two independent experiments (n = 4). Paired *t*-test (E) used to compare statistical differences. **P* < .05.

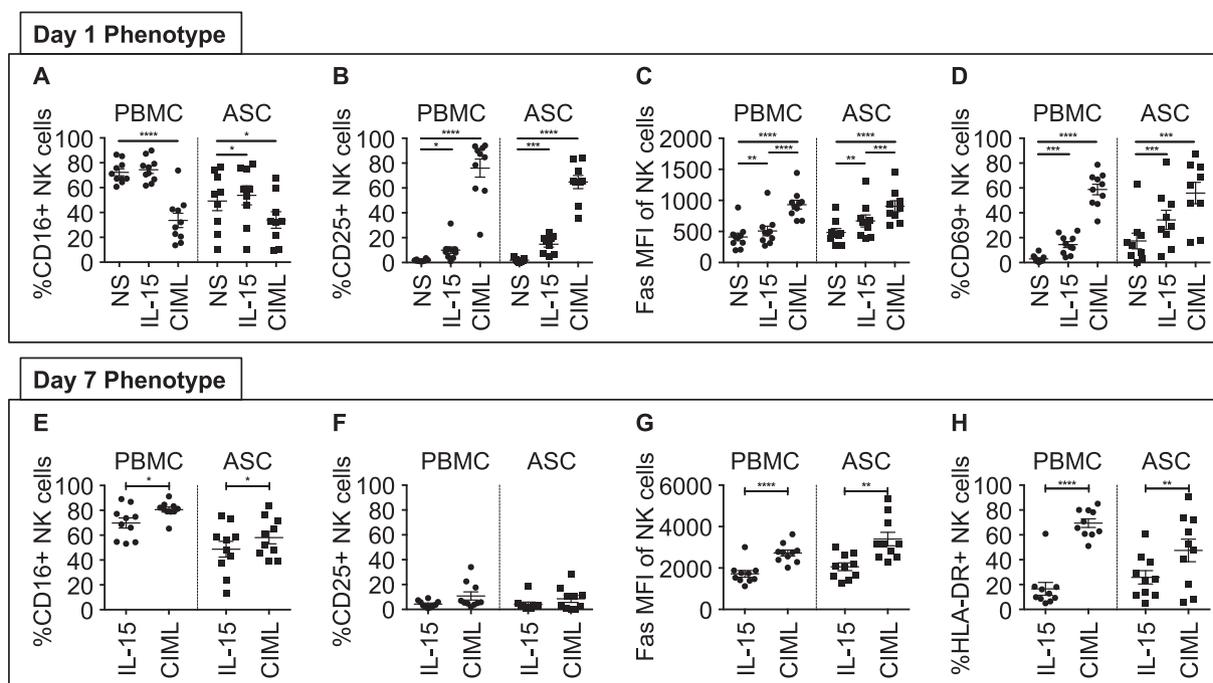


Fig. 3. Phenotypic assessment of healthy donor PBMCs and ASC of ovarian cancer patients following cytokine activation with IL-15 or IL-12, IL-15, and IL-18. Frozen PBMCs from healthy donors and ascites (ASC) cells from patients with ovarian cancer were incubated with IL-15 (1 ng/mL) or CIML conditions for 16 h, washed to remove cytokines, then placed with 1 ng/mL of IL-15 for 7 days. Phenotype assays performed at day 1 on PBMCs cells from healthy donors and ASC treated with no stimulation (NS), IL-15, or CIML NK cells gated on live, CD56⁺CD3⁻ by flow cytometry to evaluate the percentage of NK cells expressing (A) CD16, (B) CD25, and (D) CD69. NK cells were evaluated for median fluorescent intensity (MFI) of (C) Fas. (n = 10 PBMCs; n = 9 ASC; 3 independent experiments). Phenotype assays performed at day 7 between the IL-15 and CIML treated PBMC and ASC cells. NK cells gated on live, CD56⁺CD3⁻ by flow cytometry to evaluate the percentage of NK cells expressing (E) CD16, (F) CD25, and (H) HLA-DR. NK cells were evaluated for MFI of (G) Fas. (n = 10 PBMCs; n = 10 ASC; 3 independent experiments). Ordinary one-way ANOVA (A–D, line without brackets) and paired t-test (E–H) were used to compare statistical differences. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

cells against K562 leukemia cells. Similar to earlier experiments (Fig. 1A), we did not identify a difference in cytotoxicity on day 7 between CIML NK cells and control NK cells against leukemia or ovarian cancer cells (Fig. 4C).

As expected, the day 1 assay measuring IFN- γ production demonstrated a vast difference between preactivated and low-dose IL-15 controls (All groups; Fig. 4B). The enhanced IFN- γ production was confirmed on day 7 assays, although to a lesser extent for the ovarian cancer cell lines (Fig. 4D). Preactivated ASC NK cells consistently demonstrated lower IFN- γ response, suggesting a defect in the IL-12 or IL-18 pathway or the overall functionality of these cells after being removed from their immunosuppressive environment. These findings provide evidence CIML PBMC and ASC NK cells exhibit enhanced function against ovarian cancer cells.

3.5. Healthy donor PBMCs and ASC NK cells undergo higher rates of proliferation when treated with IL-12, IL-15, and IL-18

The proliferative effect of IL-12, IL-15, and IL-18 preactivation on NK cells from healthy donor PBMCs and NK cells within ASC from ovarian cancer patients was investigated by CellTrace labeling and evaluating dye dilution after 7 days in culture with the different treatments. Healthy donor PBMC and ASC NK cells preactivated with CIML conditions underwent enhanced proliferation compared to control NK cells (Fig. 5A and B). Though the proliferation was enhanced with CIML conditions, the percentage of apoptotic (Annexin V⁺) NK cells was also significantly higher in both groups, indicating expansion is accompanied by increased death (Fig. 5C). Our cell cultures demonstrated a significant increase in cell count at day 7 with healthy donor PBMC NK cells preactivated in CIML conditions, but not within the ASC cells (Fig. 5D). There were no differences noted in NK cell viability of these cultures (Fig. 5E). These results highlight the ability of IL-12, IL-15, and IL-18 to drive NK cell proliferation of healthy PBMCs, and to a lesser extent, ASC cells.

3.6. Healthy donor CIML NK cells overcome the suppressive soluble microenvironment of patients with ovarian cancer

The tumor-induced soluble microenvironment is well known to impair the proliferative and functional capacity of NK cells [20]. Therefore, proliferation and functional assays were used evaluate if CIML NK cells from healthy donor PBMCs could overcome the soluble immunosuppressive environment *in vitro*. PBMC cells were pre-activated in control or CIML conditions and incubated separately for 7 days with individual ascites supernatant samples from 5 patients with ovarian cancer. Within CIML treated PBMCs, NK cells displayed an enhanced IFN- γ response against MA148 and SKOV3 ovarian cancer cells compared to controls (Fig. S2A). Both the CD107a and IFN- γ response was substantially diminished due to the ascites supernatant. NK cell IFN- γ production within the CIML treated PBMCs was significantly increased compared to control cells after restimulation with IL-12, IL-15, and IL-18 (Fig. S2B).

Proliferation was detected in both CIML and control NK cells from PBMCs at day 7 harvest (Fig. S2C–D). High proliferation, measured as percentage of cells with 3+ divisions, was significantly increased in CIML NK cells compared to control NK cells (Fig. S2C–F). These findings suggest some of the immunosuppressive effect on NK cells within the soluble microenvironment can be overcome with CIML treatment.

3.7. Human CIML NK cells exhibit potent antitumor effects within an *in vivo* xenogeneic mouse model of ovarian cancer

Finally, to test if enriched human NK cells preactivated with IL-12, IL-15, and IL-18 could control tumor growth in a preclinical *in vivo* model of ovarian cancer, an adoptive transfer was performed within a xenogeneic mouse model of ovarian cancer (Fig. 6A). Luciferase expressing MA148 ovarian cancer cells were pre-implanted prior to the adoptive transfer and tumor progression was tracked *via* bioluminescence

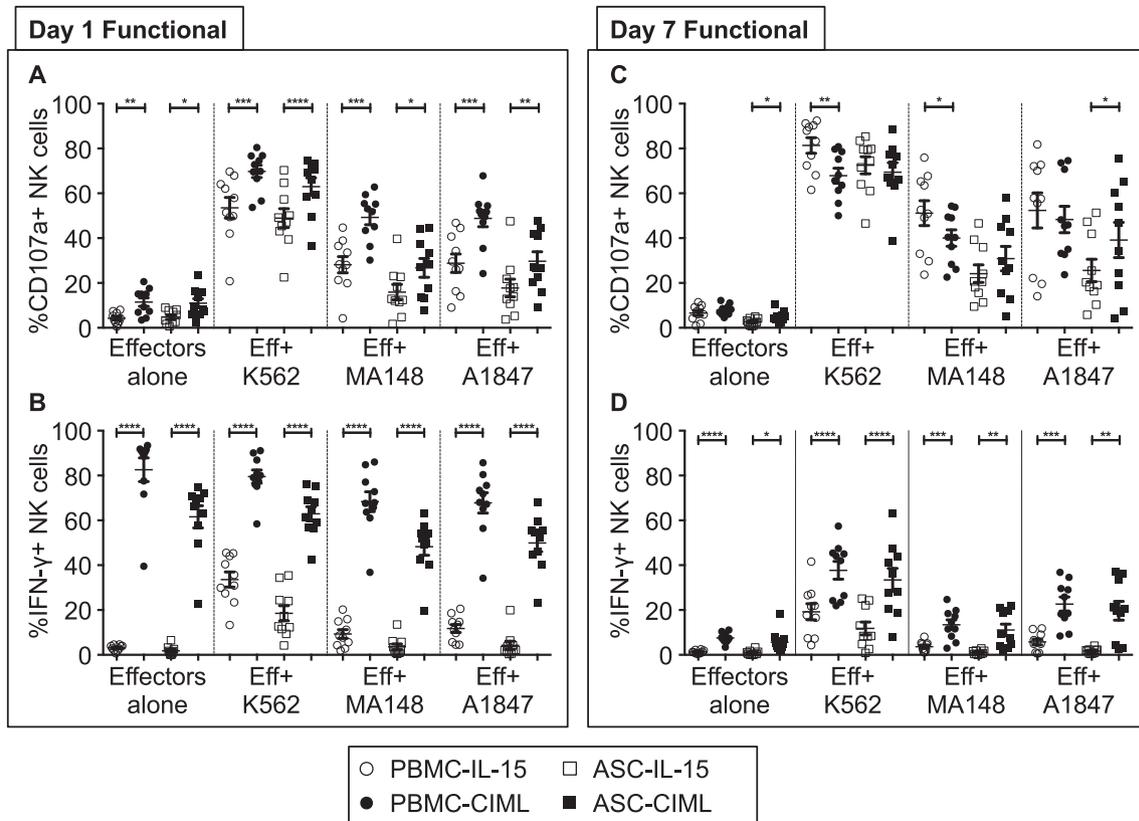


Fig. 4. CIML NK cell IFN- γ production in cryopreserved healthy donor PBMC and ASC cells. Cryopreserved PBMCs from healthy donors (circles) and ascites (ASC) cells from patients with ovarian cancer (squares) were incubated with 1 ng/mL IL-15 (white) or CIML conditions (black) for 16 h and then washed to remove cytokines. A subset of cells from each group was then placed with 1 ng/mL of IL-15 for 7 days. Functional assays were performed at day 1 and day 7. At time of functional assays, effectors were placed with K562 or ovarian tumor targets at a 2:1 effector (PBMC) to target (tumor) ratio for 4 h. Summary data shown as means \pm SEM percentage of (A) day 1 CD107a, (B) day 1 IFN- γ , (C) day 7 CD107a, and (D) day 7 IFN- γ NK cells (gated on live, CD56 $^{+}$ CD3 $^{-}$) by flow cytometry ($n = 10$ PBMCs; $n = 10$ ASC; 3 independent experiments). Paired t -test (A–D) used to compare statistical differences. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

imaging (BLI). Enriched NK cells preactivated with IL-12, IL-15, and IL-18 (CIML group) were more effective at controlling MA148 tumor growth when compared to the IL-15 or tumor only control groups (Figs. 6B and S3). Mice that received NK cells demonstrated decreased tumor growth at 6 and 13 days compared to tumor only mice. However, tumor suppression was significantly maintained past 13 days only in the mice that received CIML NK cells.

At day 25, peritoneal cells from the sacrificed mice were harvested to evaluate phenotype and function. A robust, ~ 10 -fold expansion of NK cells was identified in mice that received CIML NK cells compared to the control NK cell group (Fig. 6C). Cells harvested from mice that received CIML NK cells were associated with increased levels of NKG2A expression (Fig. 6D) and decreased mean fluorescence intensity (MFI) of CD57 (Fig. 6E), indicating these cells did not display terminally differentiated phenotype, but rather one associated with proliferation. There was a trend for a higher percentage of CD16 expression in these cells, indicating they were not completely immature (Fig. 6F). These experiments highlight the capability of CIML NK cells to expand and reduce the tumor burden within an *in vivo* xenogeneic mouse model of ovarian cancer.

4. Discussion

Motivated to make inroads into the unmet needs within ovarian cancer therapy, we investigated if short-term preactivation of human NK cells with IL-12, IL-15, and IL-18 mediated enhanced anti-tumor responses. These results provide the first evidence of enhanced anti-tumor activity of human CIML NK cells against ovarian cancer. CIML NK cells demonstrate enhanced IFN- γ production and *in vitro* killing of

ovarian cancer cells. Using the IncuCyte® Zoom platform, we found CIML NK cells demonstrate enhanced killing of ovarian cancer cells over a long period of time. Importantly, CIML NK cells retain specificity for tumor and do not demonstrate unwanted effector function against autologous cells. Moreover, CIML NK cells were superior in expansion, survival, and reducing tumor burden within a pre-clinical *in vivo* mouse model of ovarian cancer.

Our results highlight the ability of IL-12, IL-15, and IL-18 to drive proliferation of healthy NK cells. Phenotype analysis suggests CD16 is initially cleaved on cytokine-induced activation, but quickly returns to baseline levels by day 7. This decrease is in line with previous findings that strong NK cell activation can lead to temporal shedding of CD16 [31]. In addition, the expanded NK cell population appears to express a more immature phenotype with the increased expression of CD69+ and HLA-DR+. Functional analysis of CIML ASC cells demonstrated reduced IFN- γ production compared to healthy donor CIML PBMCs against leukemia and ovarian cancer cells, suggesting a defect in IL-12 and IL-18 signaling on NK cells retrieved from the ascites of ovarian cancer patients. Furthermore, our findings demonstrate some of the immunosuppressive effect on NK cells within the soluble microenvironment can be overcome with healthy CIML NK cells.

Molecular mechanisms driving the long-term functional differences identified with CIML NK cells are currently unknown. There are two competing concepts: (i) a differentiation process that results in long-term changes in molecular mechanisms, and (ii) an activation process that results in an NK cell population with enhanced activated state. Recently, Romee et al. used mass cytometry to identify distinct phenotypes of control and memory-like NK cells and these results support a differentiation process [27]. Similarly, we observed increase expression of

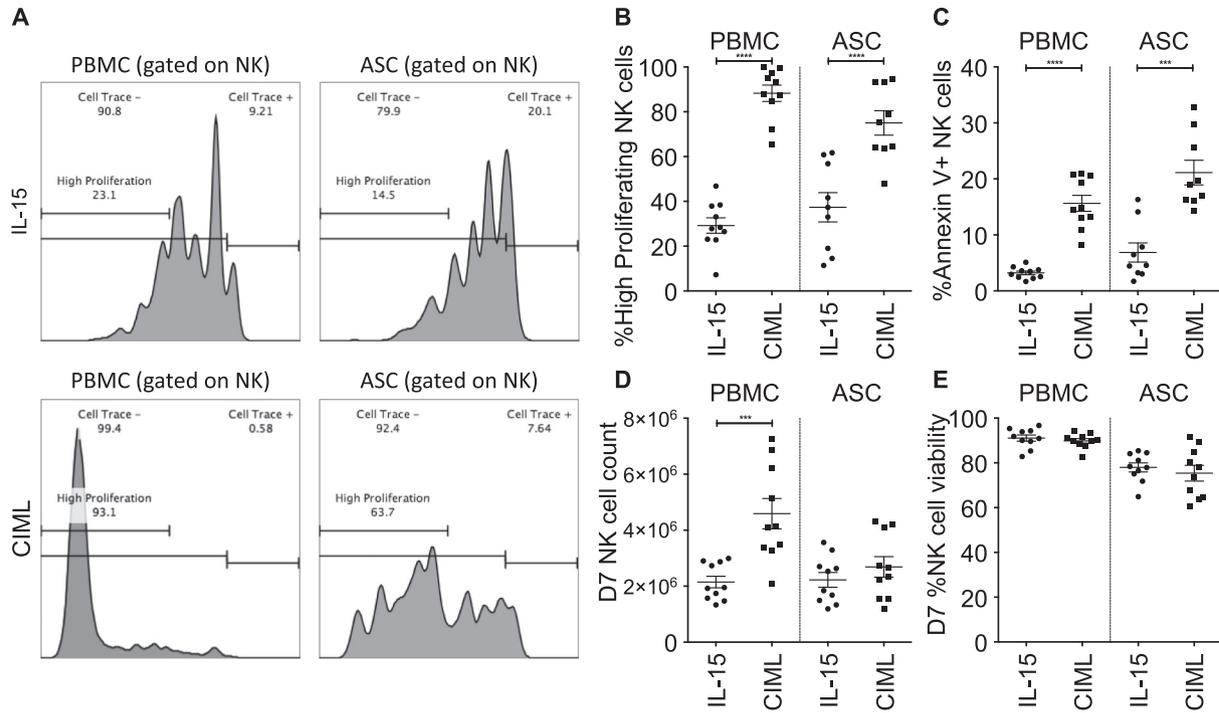


Fig. 5. Healthy donor PBMCs and ASC cells treated with IL-12, IL-15, and IL-18 undergo higher rates of proliferation. Frozen PBMCs from healthy donors and ascites cells (ASC) collected from ovarian cancer patients were labeled with CellTrace Violet dye and incubated with IL-15 (1 ng/mL) or CIML conditions for 16 h, washed to remove cytokines, then placed with 1 ng/mL of IL-15 for 7 days. Cells were then stained for surface antigens. (A) Representative histograms of CD56⁺CD3⁻ gated NK cells from healthy donor PBMC or ASC cells treated with IL-15 or CIML showing CellTrace dilution. Summary of pooled data shown as means \pm SEM percentage of (B) high proliferation (% of cells with 3+ divisions) and (C) Annexin V on PBMC NK cells (n = 10) and ASC NK cells (n = 9) donors. (D) Day 7 cell count and (E) viability of NK cells within PBMC and ASC groups at 7 days. Circles and squares represent different individual samples within groups (n = 10; 3 independent experiments). Paired t-test (B–E) used to compare statistical differences between IL-15 and CIML groups. ****P < .001; ****P < .0001.

NKG2A in NK cells following IL-12, IL-15, and IL-18 preactivation. In contrast to their study, we observed no change in CD25 (at day 7) and a decrease in CD57 expression (data not shown). The dynamic changes in receptor expression observed from day 1 to day 7 supports the differentiation hypothesis. In addition, the increased CD25 expression on day 1 followed by a return to baseline by day 7 argues against the alternative enhanced activation hypothesis. Future research should investigate these molecular mechanisms to clarify the issue.

One of the main barriers to NK cell-based adoptive cellular therapies has been obtaining a safe product that persists and demonstrates antitumor activity. Early attempts with adoptive cellular immunotherapy used autologous lymphokine activated killer (LAK) cells [32–34]. Immune cells removed from the peripheral blood of patients were activated with high-dose IL-2 and then infused back into the same patient. These studies demonstrated limited clinical benefit with high rates of peritoneal fibrosis. More recently, the adoptive transfer of cytokine-induced killer (CIK) cells against ovarian cancer has been investigated. Similar to LAK immunotherapy, CIK cells are produced from PBMC cultures with stimulation of anti-CD3 mAb, IFN- γ and IL-2 and comprise a mix of T and NK cell phenotypes [35]. A recent phase III clinical trial investigated adoptive transfer of autologous CIK cells following primary debulking surgery and adjuvant carboplatin/paclitaxel chemotherapy [36]. The results demonstrated a significant improvement in median progression free survival (37.7 months vs 22.2 months; $P = .004$) with no improvement in overall survival (61.5 months vs 55.9 months; $P = .289$). The therapy was well tolerated with no grade III or IV adverse reactions. Recent insight into the molecular mechanisms regulating NK cell function has shifted the focus towards allogeneic NK cell immunotherapy. These strategies aim to improve the antitumor activity of NK cells by mismatching donor killer-cell immunoglobulin-like receptors and recipient MHC class I molecules [37]. A phase II clinical trial investigated haplo-identical related IV infused NK cells in patients with recurrent ovarian (n = 14)

and breast cancer (n = 6) [38]. While the strategy proved safe, no successful NK cell persistence or expansion was noted, possibly due to recipient regulatory T cell expansion and reconstitution following therapy. More recently, a phase I clinical trial demonstrated the safety and feasibility of using random healthy donor-derived allogeneic NK cell therapy for advanced solid tumors [39]. Due to safety concerns of using random allogeneic NK cells, the protocol did not include immunosuppressive methods to enhance NK cell persistence.

Efforts are now underway to generate novel NK cell products that overcome the limitations identified from earlier research for adoptive transfer in the ovarian cancer setting. One example involves use of GSK3 kinase inhibition to enrich mature adaptive NK cells from CMV positive donors *ex vivo* [40]. The NK cell product demonstrates much higher production of cytokines (TNF- α and IFN- γ) and antibody-dependent cell-mediated cytotoxicity when exposed to cancer cells. A phase I clinical trial administering this product IP in women with recurrent ovarian cancer has opened at the University of Minnesota (NCT03213964). Approaches using induced pluripotent stem cell derived NK cells (with or without genetic modifications), cancer specific NK chimeric antigen receptors, and engineered bispecific antibodies and bispecific/trispecific killer engagers (BiKEs or TriKEs) are also being explored and on the way to the clinic.

In conclusion, we identified durable, enhanced functional activity of human CIML NK cells against ovarian cancer. Given these findings, we argue CIML NK cell therapy should also be considered in the ovarian cancer clinical setting. We show short-term incubation with cytokines induces greater NK cell expansion and tumor control in a preclinical model and also enhances normal donor function in a soluble ascites derived immunosuppressive environment. Given the functional differences seen between normal donors and NK cells derived from the ascites of ovarian cancer patients, the benefits discussed from allogeneic NK cell treatments, and the inherent dangers involved in treatment of patients directly with IL-12 and IL-18, one utilization of this therapy

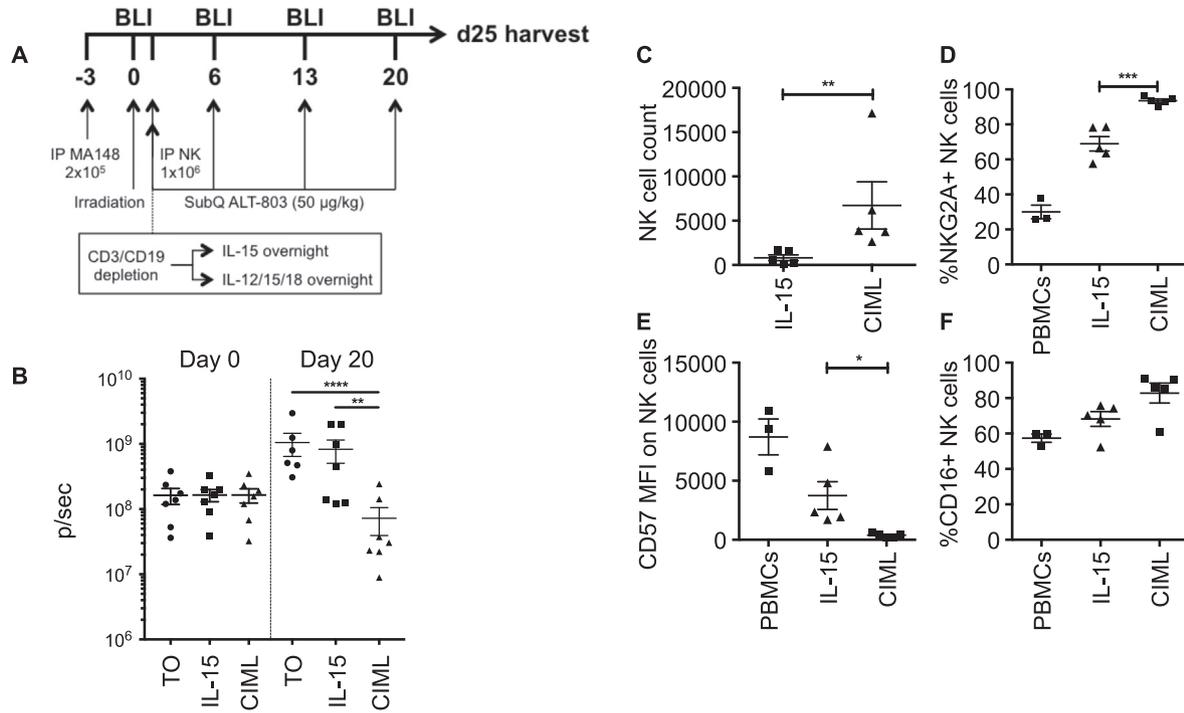


Fig. 6. Preactivation of human NK cells with IL-12, IL-15, and IL-18 decreased tumor burden within an *in vivo* xenogeneic mouse model of ovarian cancer. (A) Schema of xenogeneic ovarian cancer tumor model. NK cells from a CD3/CD19 depleted product were incubated with IL-15 (1 ng/mL) or IL-12 (10 ng/mL) + IL-15 (1 ng/mL) + IL-18 (50 ng/mL) for 16 h, followed by washing to remove cytokines. NSG mice were injected IP with one million NK cells from either the IL-15 group or the IL-12/15/18 group, followed by subcutaneous injection of ALT-803. Mice were injected with Luciferin and imaged weekly to evaluate tumor load *via* luminescence (p/s). Mice were sacrificed on day 25 to obtain peritoneal cells for phenotype and functional assays. (B) Tumor load demonstrated at day 0 and day 20 for each group ($n = 6$ for tumor only group due to death of mouse on day 18). Mice were sacrificed on day 25 to obtain peritoneal cells for phenotype assays ($n = 3$ for healthy donor PBMC controls; $n = 5$ for IL-15 and CIML group; two mice within IL-15 and one within CIML group had <100 NK cells by flow cytometry in a 60-s acquisition; one mouse within CIML group died prior to harvest). (C) Number of NK cell events assessed by flow cytometry in a 60-s acquisition. (D) NKG2A, (E) CD57 MFI, and (F) CD16 was assessed on NK cells, gated on live, CD45⁺CD56⁺CD3⁻. A two-way ANOVA (B, line without brackets) was used to compare statistical differences. Unpaired *t*-test (C–F) between IL-15 and CIML groups were used to compare statistical differences. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

would be through transfer of allogeneic NK cells treated overnight (*in vitro*) with IL-12, IL-15, and IL-18. Maintenance of the NK cells with either NCI-IL-15 or ALT-803, both currently being tested in the clinic, would enhance the efficacy of this treatment by providing extended expansion and maintenance of the CIML NK cells. In conclusion, these results provide the rationale to augment current NK cell-based immunotherapy strategies in the treatment of ovarian cancer.

Conflict of interest statement

The authors have no conflicts of interest to report.

Author contribution

LDU, MF, and MAG were involved with study conception and design. LDU, BK, LB, CR, PH, KLB, and APS were involved with the acquisition of data. LDU, MF, LB, and PH analyzed and interpreted the data. LDU, MF, MAG, and JM drafted the manuscript and critically revised it.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygyno.2019.01.006>.

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