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Intraperitoneal delivery of human natural killer cells for treatment of ovarian cancer in a mouse xenograft model

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Abstract

Background aims—There is an urgent need for novel therapeutic strategies for relapsed ovarian cancer. Dramatic clinical antitumor effects have been observed with interleukin (IL)-2 activated natural killer (NK) cells; however, intravenous delivery of NK cells in patients with ovarian cancer has not been successful in ameliorating disease. We investigated *in vivo* engraftment of intraperitoneally (IP) delivered NK cells in an ovarian cancer xenograft model to determine if delivery mode can affect tumor cell killing and circumvent lack of NK cell expansion.

Methods—An ovarian cancer xenograft mouse model was established to evaluate efficacy of IP-delivered NK cells. Tumor burden was monitored by bioluminescent imaging of luciferase-expressing ovarian cancer cells. NK cell persistence, tumor burden and NK cell trafficking were evaluated. Transplanted NK cells were evaluated by flow cytometry and cytotoxicity assays.

Results—IP delivery of human NK cells plus cytokines led to high levels of circulating NK and was effective in clearing intraperitoneal ovarian cancer burden in xenografted mice. NK cells remained within the peritoneal cavity 54 days after injection and had markers of maturation. Additionally, surviving NK cells were able to kill ovarian cancer cells at a rate similar to pre-infusion levels, supporting that *in vivo* functionality of human NK cells can be maintained after IP infusion.

Conclusions—IP delivery of NK cells leads to stable engraftment and antitumor response in an ovarian cancer xenograft model. These data support further pre-clinical and clinical evaluation of IP delivery of allogeneic NK cells in ovarian cancer.

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Keywords

bioluminescent imaging; immunotherapy; natural killer (NK) cells; ovarian cancer

Introduction

Standard therapies for recurrent ovarian cancer provide a dismal (<20%) response rate (1). There is an urgent need for novel therapeutic strategies because most women with relapsed ovarian cancer will die of progressive disease. Harnessing immune cells to treat malignancies has become more widely used for diverse tumors, but has not yet been shown to be effective for ovarian cancer. Natural killer (NK) cells are a key part of the innate immune system, with the ability to recognize and kill diverse types of tumor cells, including ovarian cancer (2). T-cell–based therapy has produced objective responses in metastatic melanoma; however, recurrent disease after T-cell therapy is common. In contrast to T cells, NK cells (defined by the $CD3^{-}/CD56^{+}$ phenotype) are capable of lysing virally infected cells or tumor cells without prior sensitization and are not restricted by variations in human leukocyte antigens (HLA). In clinical trials at our institution, dramatic anti-tumor effects have been seen with interleukin (IL)-2 activated NK cells that are adoptively transferred into patients with refractory leukemia (3). Importantly, clinical efficacy in these patients correlated with *in vivo* NK cell persistence and expansion. We have recently completed a phase II trial of NK cell infusions in patients with ovarian cancer (4). Although the approach is promising, limitations have been identified. Unlike treatment of leukemia, there was limited persistence and no *in vivo* expansion of intravenously (IV) delivered NK cells in patients with ovarian cancer. In the present study, we investigated the hypothesis that NK cell delivery mode contributed to the lack of *in vivo* persistence and expansion experienced clinically when allogeneic NK cells were delivered IV. We developed an ovarian cancer xenograft model to determine if the route of NK cell delivery is a major obstacle in obtaining clinical responses in ovarian cancer. We found that IP delivery leads to sustained NK cell engraftment and antitumor response. These data provide novel evidence for the ability of intraperitoneally (IP) delivered NK cells to not only inhibit tumor growth but to persist *in vivo* and to traffic to the periphery and secondary lymphoid organs. The present findings will stimulate further preclinical studies leading ultimately to clinical validation of IP NK cell immunotherapy, with the potential to affect clinical treatment in ovarian cancer.

Methods

Generation of firefly luciferase/green fluorescent protein–positive ovarian cancer cells

K562 and OVCAR-3 cells were obtained from American Type Culture Collection. The ovarian cancer cell line MA-148 cells were kindly provided by Sundaram Ramakrishnan (University of Minnesota, Minneapolis, Minnesota, USA). Luciferase and green fluorescent protein (GFP)-expressing MA-148 cells were generated with the use of a bicistronic pKT2 *Sleeping Beauty* cassette (5); 500,000 MA-148 cells were nucleofected with 1 μg of pKT2 plasmid containing a GFP:zeocin fusion protein and firefly luciferase as well as 1 μg of SB100X transposase with the use of the 4D-Nucleofector system (Lonza). Cells were passaged in zeocin-containing media and sorted with the use of a FACSAria (BD Biosciences).

Cells and mice

Peripheral blood mononuclear cells were isolated from 3- to 5-h lymphapheresis products drawn from normal donors on the day before cell infusion. Mononuclear cells were first isolated from apheresis products through density gradient centrifugation. NK cells were

enriched by depleting $CD3^+$ and $CD19^+$ with the use of magnetic beads (Miltenyi Biotec, Auburn, CA, USA). Use of peripheral blood mononuclear cells s from donors was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. After CD3/CD19 depletion, cells were activated overnight with 100 Units/mL of IL-2 (Chiron). Cells were then harvested and injected IV (Supplementary Figure 1 only) or IP into mice (day 0). Five days before (day −5) NK cell injection, NOD/SCID/$\gamma c^{-/-}$ mice were sublethally irradiated (225 cGy) and xenografted with firefly luciferase expressing MA-148 tumor cells (day −4). After tumors were engrafted for 4 days, mice were given 20×10^6 cells from the CD3/CD19-depleted and activated product. Mice then received IP injections of IL-15 (100 ng per injection) or IL-2 (75,000 units per injection). IL-2 or IL-15 was given every day for the first 7 days, followed by injections every Monday, Wednesday and Friday for 3 additional weeks. Before NK cell injection (day −1) and on days 7, 14, 21, 40 and 53, mice were analyzed for the presence of tumor cells by means of BLI, with the use of the Xenogen IVIS Imaging system (Caliper Life Science, Hopkinton, MA, USA).

Antibodies and flow cytometry

The following antibodies were used: CD45-PE, CD56-APC, CD16-PercpCy5.5 and CD117-PercpCy5.5, all from Becton Dickinson. CD158a/h-PE, CD158j-PE, CD158i-PE, CD158e1/e2, CD159a-PE and -APC were obtained from Beckman Coulter. Flow cytometry was performed on a BD FACS Calibur or LSRII, and data were analyzed with the use of FlowJo (Treestar).

In vitro cytotoxicity

Tumor targets (K562, OVCAR3 and MA-148) were incubated with ^{51}Chromium for 1 h at 37°C, washed three times and co-cultured with NK cells at indicated effector to target (E:T) ratios. Total lysis (test release) was achieved with the use of 5% Triton-X 100. After a period of 4 h, cells were harvested and analyzed. Specific ^{51}Chromium lysis was calculated by means of the equation

$$\%\text{Specific lysis} = 100 \times (\text{test release minus spontaneous release}) / (\text{maximal release minus spontaneous release})$$

In vivo evaluation of NK cell persistence

On days 7, 14 and 21, mice were retro-orbitally bled for evaluation of *in vivo* persistence; 100 μL of blood was collected, lysed, blocked with 0.5% human AB serum (Valley Biomedical) and stained for the presence of surface antigens present on human NK cells. At day 54 after NK cell injection, mice were euthanized to evaluate IP tumor burden as well as NK cell engraftment in the spleen and peritoneum. As above, cells were blocked and stained for the presence of various surface markers present on NK cells. Peritoneal washes were also evaluated for the presence of MA-148 tumor cells, which express GFP.

Ex vivo expansion of engrafted splenic NK cells

At the time of euthanasia, each spleen was split in half, in which one half was analyzed by flow cytometry and the other half was expanded *ex vivo* through the use of artificial antigen-presenting cells (aAPCs). The presence of human NK cells within the spleen was determined by flow cytometry gating on $CD56^+CD45^+$ cells within a lymphocyte gate. This percentage was applied to the absolute count of cells recovered from the spleen sample for expansion. Each individual sample was then stimulated with aAPCs expressing membrane-bound IL-21 at a ratio of 2:1 (two aAPCs:one NK cell), which are known to markedly expand human NK cells *ex vivo* (6). Cells were fed every 3 days as previously described and

re-stimulated with aAPCs every 7 days. After 2 weeks of expansion, cells were tested against K562 and MA-148 targets in a standard chromium release assay.

Statistical analysis

Differences between groups were compared with the use of a paired t test or one-way analysis of variance with *post hoc* Tukey's test in Prism 4 (GraphPad Software, San Diego, CA, USA). Results were considered significant at values of $P \leq 0.05$.

Results

IP delivery of allogeneic peripheral blood NK cells for treatment of ovarian cancer

Peripheral blood NK cells can be easily enriched from allogeneic donors for adoptive immunotherapy. This has been successfully been used for therapy in refractory AML (3); however, NK cell immunotherapy has been less effective in solid tumors (7,8). We hypothesized that deficiencies of adoptively transferred NK cells *in vivo* were not caused by their inability to kill ovarian cancer cells but by their failure to effectively traffic from circulation to the tumor site. We first confirmed the ability of activated donor allogeneic peripheral blood (PB)-NK cells to kill ovarian cancer cells *in vitro*. Donor PB-NK cells were isolated and prepared as described (Methods section) (Figure 1). As opposed to enrichment with the use of anti-CD56 microbeads for selection of NK cells, we found that a CD3/19 depletion leads to fewer NK cells lost during processing and allows higher absolute numbers for infusion (3). In standard cytotoxicity assays, we found both resting and IL-2–activated (100 U/mL) PB-NK cells were able to kill the NK cell–sensitive target K562 to similar levels. However, IL-2–activated PB-NK cells performed significantly better against the ovarian cancer targets MA-148 ($P = 0.01$) and OVCAR3 ($P = 0.03$) (Figure 1). This is most probably caused by upregulation of several surface effector molecules (CD 16, NKp46, NKG2A/CD94, KIR and NKG2D, perforin) after cytokine stimulation, as has been demonstrated in other studies (9–11).

Because the NK cells are capable of recognizing and killing ovarian tumor targets *in vitro*, we hypothesized that delivery of anti-tumor lymphocytes directly to the site of tumor origin would overcome the defects in NK cell trafficking into the peritoneum. Ovarian cancer provides an optimal model to study this because we can position both the tumor targets and effector NK cells in murine recipients similar to the metastatic disease found in humans, in which the primary route of disease spread is direct seeding throughout the peritoneal cavity. We previously found that IV delivery of NK cells does not halt ovarian cancer growth in an *in vivo* model (Supplementary Figure 1) or in patients in our clinical trial (4). Although IP delivery of NK cells directly to the peritoneum appeared as an appropriate next step in the treatment of this disease, it has never been demonstrated that direct delivery of peripheral blood NK cells to the peritoneum would be capable of stable long-term cell survival and anti-tumor activity.

IP delivery of NK cells leads to stable engraftment and anti-tumor response

We next tested whether or not IP delivery of PB-NK cells had any benefit compared with IV delivery. Our main focus was to measure levels of NK cell engraftment within the periphery to verify that IP delivery of NK cells would provide circulating antitumor effectors. Interestingly, we found that NK cells given IP had levels of circulating NK cells similar to those delivered IV (Supplementary Figure 2). We also found high numbers of NK cells in the spleen and bone marrow weeks after delivery (Supplementary Figure 2), without apparent differences in the absolute number of cells recovered between the two groups. These results demonstrate that IP-delivered NK cells are capable of circulating to the periphery and secondary lymphoid organs and could be capable of controlling metastatic

disease, because ovarian cancer cells have been shown to circulate in the peripheral blood (12).

Although IP delivery of human NK cells leads to stable engraftment, we aimed to demonstrate that this was an effective means to clear IP tumor burden. To test this in an *in vivo* model, we stably modified MA-148 ovarian tumor cells with a *Sleeping Beauty* cassette carrying both GFP and firefly luciferase reporter genes. We then devised an IP therapy model on the basis of our previous clinical experience for adoptive transfer of NK cells to patients with ovarian cancer. We administered 200,000 luciferase-expressing MA-148 cells to NOD/SCID/$\gamma c^{-/-}$ immunodeficient mice IP and allowed them to engraft for 4 days. Mice then received 20 million PB-NK cells IP. IL-2 and IL-15 were given as described in the Methods section. Tumor burden was followed with the use of bioluminescent imaging (BLI). Cytokine alone does not have an anti-tumor effect (unpublished data). Two weeks after NK cell delivery, we could see marked tumor reduction in mice receiving IP NK cells compared with tumor-only controls (Figure 2). Each mouse inoculated with tumor but not receiving NK cells exhibited tumor growth, whereas mice receiving NK cells had lower levels of tumor burden, with only the IL-2 group demonstrating significantly decreased tumor burden. Given these data, we conclude that IP delivery of NK cells is an effective method for anti-tumor therapy, leading to both stable engraftment and disease regression.

IP-delivered NK cells persist in vivo while maintaining a mature phenotype

We followed the mice for disease progression and peripheral engraftment for approximately 8 weeks. At day 54, we euthanized the mice to evaluate NK cell engraftment within the peritoneum and organs. After mice were euthanized, we performed peritoneal washings with the use of sterile phosphate-buffered saline to recover any free-floating cells (lymphocytes and/or tumor cells). In mice receiving IP infusions, there were high percentages of NK cells within the peritoneal cavity at time of euthanasia. The mice receiving IL-15 alone had lower percentages of NK cells (Figure 3). This finding is most probably caused by the dose of IL-15 used, which is subtherapeutic for long-term persistence. In these experiments, we used a dose of 100 ng per mouse. However, other recent studies are using between 2–5 mg per mouse to show successful *in vivo* expansion (13).

Although we observed persistence of NK cells in the mice, there remained signs of localized tumor burden on BLI and at necropsy (Supplementary Figure 3). BLI of each group before euthanasia (day 53) demonstrated significantly reduced tumor burden in the mice receiving PB-NK cells with IL-2 or IL-15 alone (Supplementary Figure 3). Interestingly, we found more profound effects of IP-injected NK cells on tumor burden through analyzing peritoneal washes for residual ovarian cancer (tumor cells expressing GFP). We were surprised to find that peritoneal tumor burden in mice receiving NK cells and IL-2 was significantly reduced to the level of the negative control. Whereas the tumor-only mice had high levels of peritoneal tumor burden (34.3% ± 8.426% GFP^+ MA-148 tumor cells), NK cell–treated mice had markedly reduced levels, with the IL-2 group having 0.375% ± 0.144% and the IL-15 group having 19.11% ± 9.17%, probably caused by the lower levels of NK cells (Figure 4). These data strongly support the hypothesis that IP delivery of NK cells is effective at clearing ovarian cancer within the peritoneal cavity, because most tumor burden was located in subcutaneous tissue consistent with tumor track infiltration. It remains possible that IP-injected NK cells are fully capable of clearing ovarian cancer cells but may not traffic to extra-peritoneal sites because of their activated phenotype after IP injection of cytokines.

Persisting NK cells remain functional and have markers of maturation

Although the NK cells present had markers indicating their functionality (KIR, NKG2A), we could not rule out the possibility that persisting NK cells were hypo-responsive. To test this, we aimed to evaluate the function of NK cells *ex vivo*. First, we had to overcome the low yield and absolute numerical differences between mice. Several groups have used aAPCs to expand both NK cells and T cells (6). By calculation of the percentage of human NK cells in each mouse spleen, samples were stimulated with aAPCs at a 2:1 ratio, as previously demonstrated (6). We found that this is an effective method to expand NK cells for further *in vitro* testing. Samples from each mouse, although varied in their initial starting cell number, expanded at an equal rate to levels, far exceeding our expectations (Figure 5). On average, each sample expanded approximately 100-fold. This allowed for further phenotypic and functional testing. Although the input cells varied in their phenotype, we found that expansion with aAPCs restored NK cells to baseline level, demonstrating high levels of CD56 expression as well as KIR and CD16. These data strongly support the *in vivo* phenotype and functionality of the persisting NK cells. We proceeded to test these cultures against both K562 and MA-148 targets by use of a standard chromium cytotoxicity assay and found that each sample was effective at killing both targets similar to or higher than their pre-infusion levels (Figure 5). These findings further support our hypothesis that *in vivo* functionality of human NK cells can be maintained and they do not become exhausted.

Given the long-term persistence of functional NK cells, we tested the expanded cells for markers of maturation. After 2 weeks of expansion, we found that approximately one third of the NK cells expressed the activating receptor NKG2C, with a smaller proportion expressing the terminal maturation marker CD57 and CD158b (Figure 6). Nearly all of the NK cells expressed the inhibitory receptor NKG2A, which can be upregulated in response to cytokines. The CD57, NKG2C and CD158b markers are characteristic of mature NK cells, and, in conjunction with our functional data, supports the idea that NK cells, commonly thought of as shortlived, innate lymphocytes, could serve as a promising anti-ovarian cancer treatment when delivered IP.

Discussion

Standard therapies for recurrent ovarian cancer provide a dismal response rate (1). There remains a critical need for novel therapeutic strategies because most women with relapsed ovarian cancer will die of progressive disease. Our study focuses on the use of an immunotherapeutic strategy, specifically harnessing NK cells to kill ovarian cancer cells (4,14). NK cells function to kill ovarian cancer cells through activating receptors in the context of missing self-ligands (cognate HLA molecules) downregulated on ovarian cancer targets (4,8,14). We have previously initiated preclinical studies and clinical trials with the use of peripheral blood NK cells. As opposed to magnetic enrichment of $CD56^+$ NK cells, we found that a CD3/19 depletion leads to less loss of NK cells during processing and allows higher absolute numbers for infusion. Although this rids most of the harmful $CD3^+$ T cells and $CD19^+$ B cells, there remain a large proportion of $CD14^+$ monocytes. Our clinical trials show that the majority of donor cells that do engraft and survive are $CD56^+$ NK cells (3), though we cannot fully rule out a role for monocytes in our model or patients. Although monocytes are typically short-lived cells, we did not assess for them in the peritoneum of mice. We did find a large number of $CD45^+CD56^-$ cells within the spleen and bone marrow of mice injected IP with NK cells, indicating a population of human blood cells that are not NK cells (Supplementary Figure 2). This could contain a population of tissue-resident monocytes/macrophages. There have been studies that have shown a population of $CD56^+$/$CD14^+$/HLA-DR^+ accessory dendritic cells; however, our cells do not co-express these markers in the mouse model or in patients (15). These accessory dendritic cells have been shown to positively influence NK cell activity, and it remains possible that the presence of

monocytes in our NK cell product is beneficial. Further studies elucidating the role and potentially harnessing the activity of these cells are needed.

Although NK cells are active against ovarian tumors *in vitro*, their activity *in vivo* has been limited (4). Lack of *in vivo* activity can be correlated to several clinical findings. First, to date, we have been unable to achieve successful NK cell expansion *in vivo* in an ovarian cancer population (4,14). The use of doses of IL-2 sufficient to expand adoptively transferred NK cells in AML fails to expand NK cells in patients with ovarian cancer beyond 14 days after IV NK cell infusion. The reasons for lack of expansion may be multifactorial. One reason could be that the preparative regimen used in the ovarian cancer trials does not allow for sufficient “cleared space” for successful expansion of adoptively transferred lymphocytes. Alternatively, after lymphodepleting chemotherapy, there is a high level of T-regulatory cell (Treg) expansion. The Tregs are of recipient origin and further support the insufficient level of immunosuppression preceding adoptive transfer. Tregs are capable of outcompeting NK cells for the exogenous IL-2 through their constitutive expression of the high-affinity IL-2 receptor. It is also known that immunosuppressive cytokines such as transforming growth factor-β are elevated in patients with ovarian cancer, which may further lead to the development and expansion of Tregs (16–18). Transforming growth factor-β also blocks the anti-tumor activity of NK cells in ovarian cancer (18).

It remains unknown whether the traditional IV route of delivery of adoptively transferred NK cells is optimal in patients with ovarian cancer. Little is known about the natural biology of NK cell trafficking, and it is unlikely that NK cells administered IV receive the proper signals to traffic into the peritoneum of patients with ovarian cancer. We know from our previous clinical experience that IV-administered NK cells have not been effective, to date (4). Because NK cells are highly effective at killing ovarian cancer cells *in vitro* (2), we therefore reasoned that NK cells maintain the intrinsic capacity to kill ovarian cancer cells *in vivo* but face limitations such as poor expansion and trafficking to the tumor site. Our studies sought to overcome these limitations by testing the ability of IP-injected NK cells to treat ovarian cancer. No published reports have demonstrated this approach as effective with the use of human peripheral blood NK cells, though one group delivered irradiated NK cells lines IP for antitumor immunotherapy (19). In comparing the differences between IV versus IP PB-NK cells, we were surprised to find that IP delivery of lymphocytes led to similar levels of engraftment in all organs tested, including peritoneum, peripheral blood, spleen and bone marrow. This indicates that IP adoptively transferred NK cells are not only capable of *in vivo* engraftment but maintain the ability to traffic to the circulation. By use of an *in vivo* xenograft model of ovarian cancer, we were able to show the activity of IP-transferred NK cells. NK cells injected directly into the peritoneum of mice with previously established ovarian tumors were able to limit disease compared with noninjected controls (Figures 2 and 4). Most surprisingly, at time of necropsy, mice receiving IP-delivered NK cells in combination with IL-2 injections had no peritoneal disease, indicating potent and sustained activity of IP-delivered NK cells. Mice not receiving NK cells had marked ascites and tumor burden, as indicated by necropsy and BLI. The remaining disease burden was outside the peritoneum, primarily in the subcutaneous tissue. This was probably caused by seeding from repeated injections. This is a common occurrence clinically and often requires local excision.

Lack of complete tumor clearance in animals did not appear to be caused by intrinsic inability of the NK cells to recognize and kill tumor targets. After *ex vivo* expansion, adoptively transferred NK cells recovered high levels of CD56 expression as well as the effector molecules CD 16 and KIR. These are the first studies to demonstrate this duration of persistence for adoptively transferred NK cells within an *in vivo* tumor model. We believe that in opposition to current thinking, NK cells can be long-lived, as recent studies in

mice and humans have suggested (20—22). We tested whether NK cells in this system had markers of persistence and found that they indeed expressed markers of terminally differentiated NK cells (23) expressing CD57, NKG2C and CD 158b. Although these data do not functionally support the potential for NK cell memory in this model, it would be interesting to know if NK cells could be manipulated to gain this function in patients to treat diseases with high rates of recurrence, such as ovarian cancer. It is also possible that an *ex vivo* expansion model could be efficacious in patients in whom NK cells could be recovered and expanded *ex vivo* for re-infusion, thereby increasing the effector-to-target ratio until a patient is cleared of their disease.

These data provide evidence for advancing clinical trials in women with ovarian cancer. We believe that IP delivery of NK cells provides an optimal platform of immunotherapy in this disease. Because patients today often are treated with IP chemotherapy, this seems the next appropriate step. By overcoming the inability to expand adoptively transferred lymphocytes in this setting, direct delivery of effector cells to the tumor site has the potential to provide more rapid clinical responses. Additionally, we have shown the ability of NK cells to traffic to extraperitoneal sites, demonstrating the similarities in engraftment between IV and IP administration of NK cells. Our findings also suggest that patients would benefit from IP delivery of exogenous cytokines such as IL-2. The recent advancement of IL-15 also holds intense promise for such an approach. Low-dose IL-15 (100 ng/mL) was subtherapeutic in our studies and unable to halt tumor progression. Because the model does not completely recapitulate our human patients, the beneficial effect of the use of IL-15 in clinical trials could not be fully established. We hypothesize that IL-15 would be superior to IL-2 in the treatment of patients with ovarian cancer with allogeniec NK cells because IL-15 will not expand recipient Tregs (13,14,24). The presence of recipient human Tregs is not part of our model but could be performed in future studies. It has been shown that Tregs can inhibit the activity of NK cells directly and indirectly (25,26). IL-15 also led to maintenance of CD56 expression on cells in both the peritoneum and the spleen, in which IL-2–injected mice had lower CD56 expression (Figure 3). The downregulation of CD56 on NK cells is common in chronic viral infections (27). It is possible that high doses of IL-2 in this model leads to downregulation of CD56; however, the functional consequence of this down-regulation is not known (27,28). We have previously shown *in vitro* that IL-15 is required for maintenance of CD56 expression (29).

Other strategies that influence NK cell trafficking may be used as well. Although we have shown that IP delivery of NK cells allows for peripheral engraftment, blocking lymphocyte egress from the peritoneum would theoretically allow NK cells to persist IP in higher percentages, optimizing the anti-tumor response. Clinical trials have shown that reagents such as sphingosine-1-phosphate receptor modulators could be a promising avenue to this approach (30).

These data provide evidence in support of a readily translatable approach to immunotherapy for patients with ovarian cancer. IP infusion of NK cells may overcome immune barriers of adoptive transfer differently than IV infusion. The above studies provide the basis for the development of further strategies to manipulate the NK cell product, host and targets, with the ultimate goal of enhancing the therapeutic benefit of NK cell–based immunotherapy while minimizing the risks and toxicities for women with ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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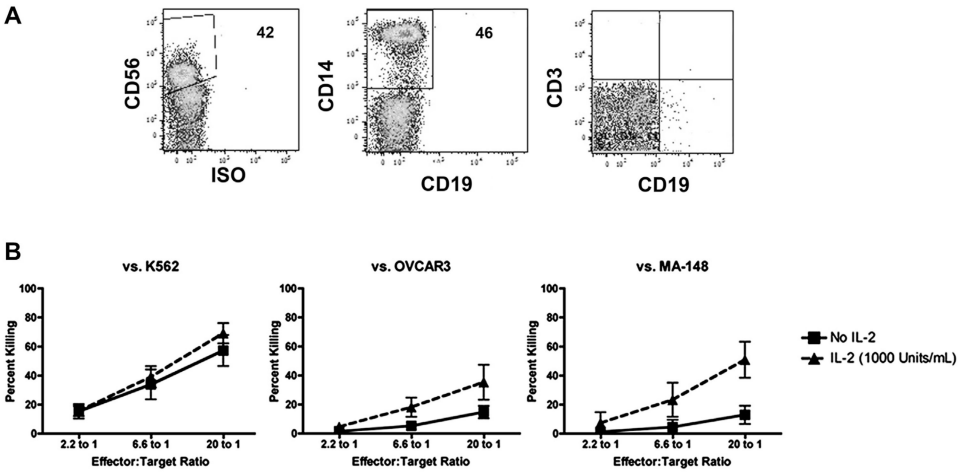

Figure 1.

Activity of allogeneic NK cells against ovarian cancer targets. (A) Representative example of the post–CD19 and CD3 depletion product for infusion. After depletion with CD3 and CD19 magnetic beads, there were minimal $CD3^+$ T cells or $CD19^+$ B cells and high percentages of $CD56^+$ NK cells and $CD14^+$ monocytes. Human hematopoietic cells were first gated with the use of the pan-hematopoietic marker CD45 (not shown). $CD45^+$ events were then gated to demonstrate the proportion of cells expressing CD56 (versus isotype), CD14 versus CD19 and CD3 versus CD19. (B) Allogeneic PB-NK cells are active after CD3/19 depletion. Overnight resting (squares with solid lines) and activated (triangles with dashed lines) NK cells were tested for their cytotoxicity against the leukemia target K562 or the ovarian cancer cell lines MA-148 and OVCAR3. IL-2–activated PB-NK cells performed significantly better against the ovarian cancer targets MA-148 ($P = 0.01$) and OVCAR3 ($P = 0.03$).

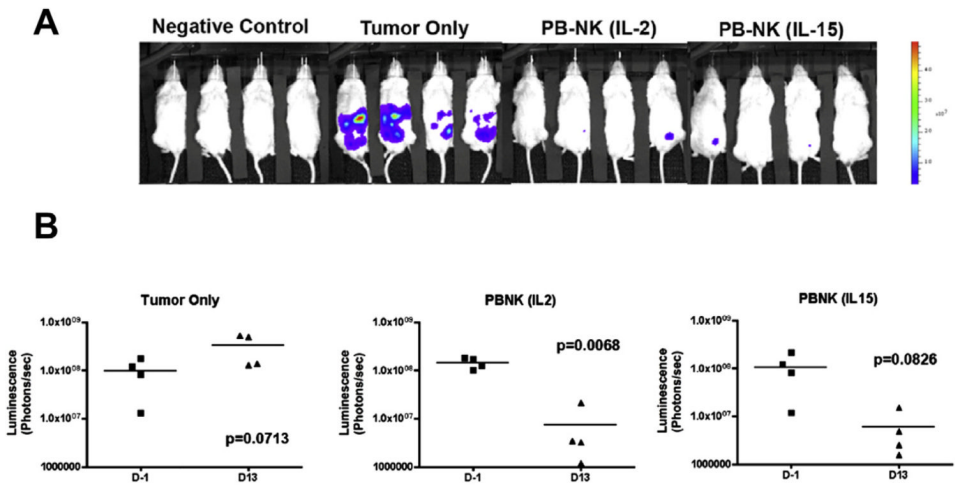

Figure 2.

Intraperitoneal delivery of NK cells leads to decreases in tumor burden. (A) Tumor burden was measured by means of BLI 13 days after infusion of NK cells. (B) Quantification of BLI signals demonstrates that the tumor-only group had an increase in tumor burden. Only the PB-NK (IL-2) treatment group demonstrated a significant reduction in tumor signal (P = 0.0068) at day 13 versus day −1. The reductions in signal in the PB-NK (IL-15) group was nonsignificant (P = 0.0826). Differences within groups were determined by means of a paired Student's t test.

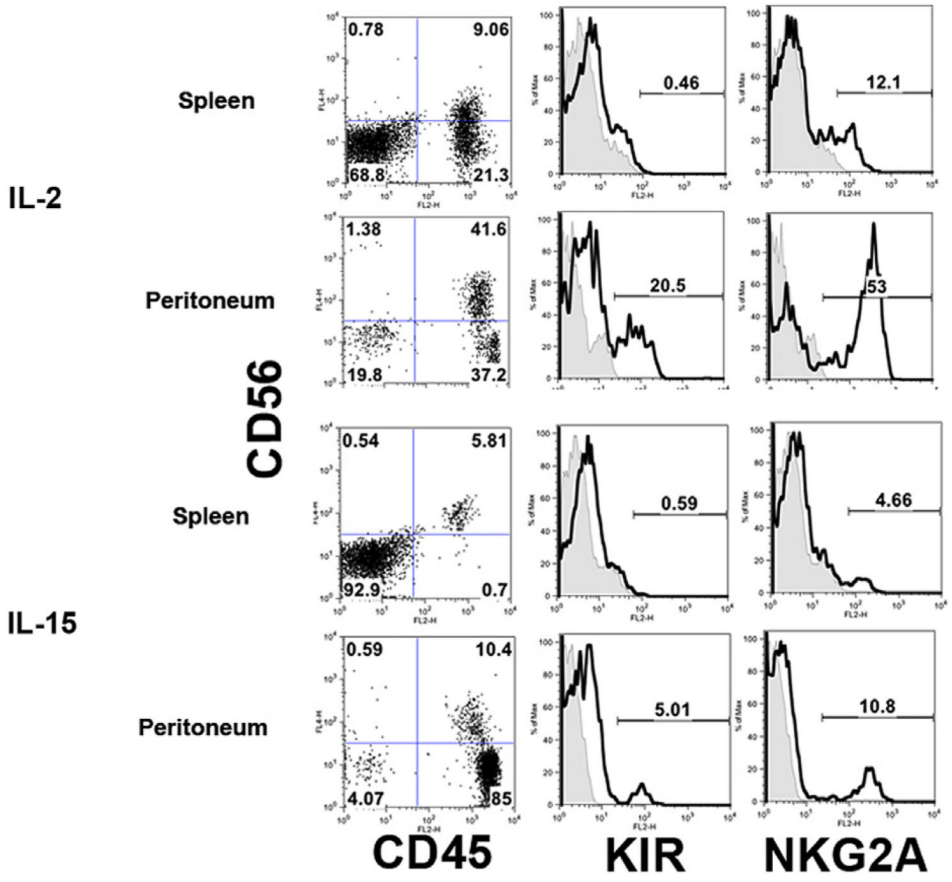

Figure 3.

Engraftment analysis at day 54 after NK cell injection. At the time of euthanasia, mice were analyzed for engraftment of NK cells within the spleen and peritoneal cavity. Human NK cells were identified by their expression of CD56 and CD45. $CD56^{+}$ cells were further analyzed for the expression of killer immunoglobulin–like receptors and NKG2A. Representative results of a single mouse are shown.

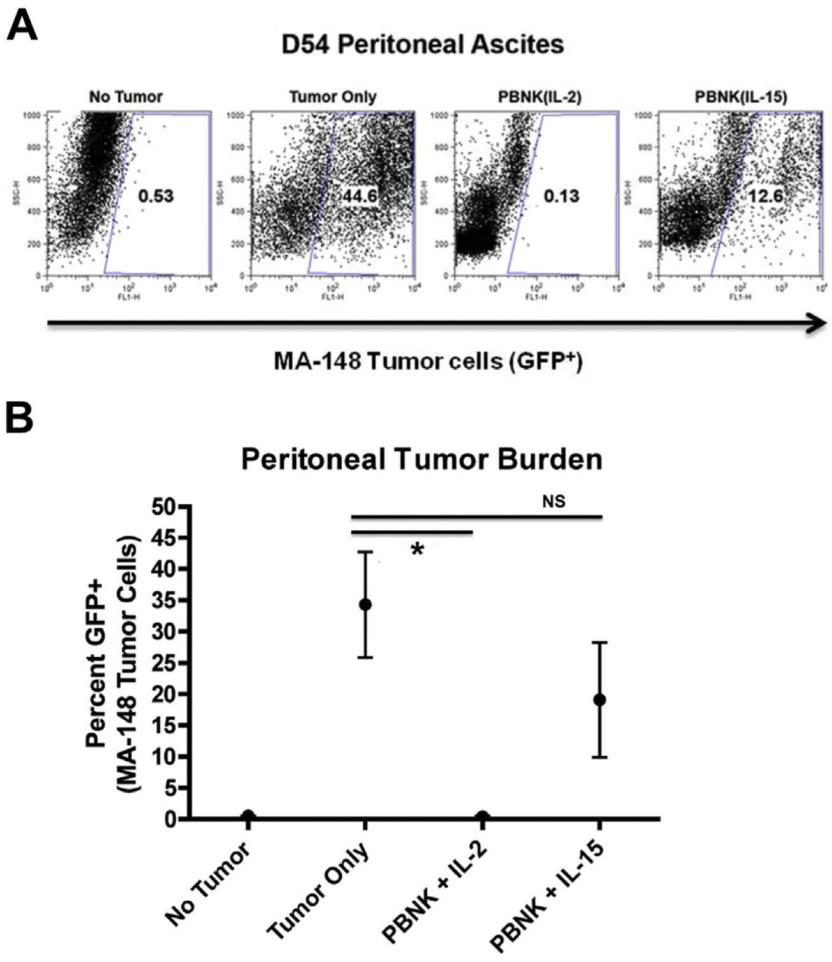

Figure 4.

Peritoneal tumor burden at day 54 after NK cell injection. (A) At the time of euthanasia, mice were analyzed for tumor burden within the peritoneal cavity. Peritoneal washes were performed with the use of sterile phosphate-buffered saline. Cells were then analyzed by flow cytometry for the presence of GFP^+ MA-148 tumor cells. Representative plots of each treatment group are shown with quantification in (B). Differences between groups were analyzed with the use of a one-way analysis of variance. There was a significant difference between all groups [$F(3,10) = 6.073$, $P = 0.0127$]. A *post hoc* Tukey analysis comparing each individual treatment group to tumor-only controls demonstrated a significant effect with the PB-NK (IL-2) treatment group ($P < 0.05$ indicated by asterisk). There was no significant difference between the tumor-only and PB-NK (IL-15) or between PB-NK (IL-2) versus PB-NK (IL-15) treatment groups. NS = nonsignificant.

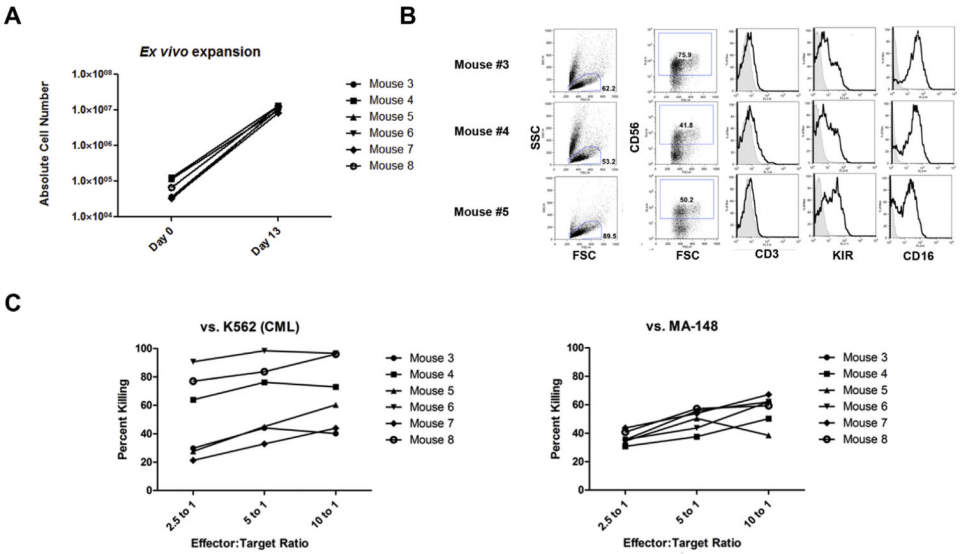

Figure 5.

Ex vivo expansion, phenotype and function of splenic NK cells. (A) After analysis of splenic NK cell engraftment, cells were stimulated with aAPCs expressing mbIL-21 (three mice per group). After 13 days of expansion (two stimulations), each mouse sample was quantified and analyzed. (B) Representative flow cytometry plots from two individual mice are shown. After 13 days of expansion, there were numerous $CD56^+$ NK cells. Gating on $CD56^+$ cells demonstrates high co-expression of CD16 and killer immunoglobulin–like receptors without any human $CD3^+$ T cells. Dark black lines in histogram represent the individual stains compared with an unstained control (shaded gray). (C) Each expanded line was then tested for their ability to kill K562 or MA-148 cells in a chromium-release cytotoxicity assay.

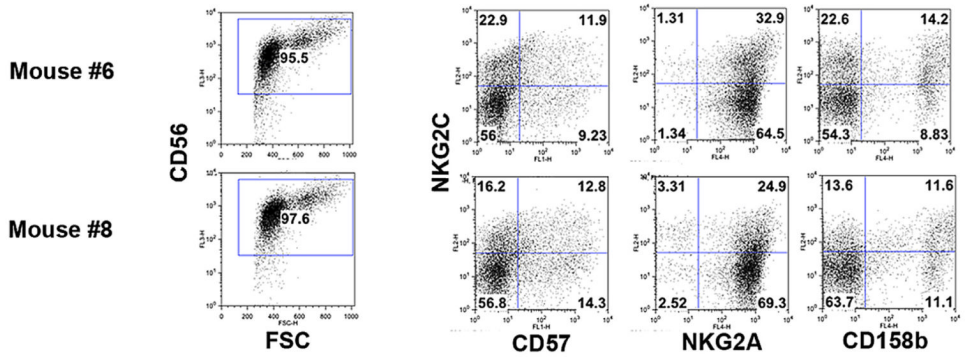

Figure 6.

Long-lived, persistent NK cells have markers of maturity. After expansion, NK cells recovered from the spleen were measured for markers of maturity. Representative mice demonstrate that each group demonstrates high levels of mature NK cell markers. $CD56^{+}$ NK cells were analyzed for co-expression of the markers NKG2C, CD57, NKG2A and CD158b.