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NATURAL KILLER CELL THERAPY AND AEROSOL INTERLEUKIN-2 FOR THE TREATMENT OF OSTEOSARCOMA LUNG METASTASIS

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Abstract

PURPOSE—Survival of patients with osteosarcoma lung metastases has not improved in 20 years. We evaluated the efficacy of combining natural killer [NK] cells with aerosol interleukin-2 [IL-2] to achieve organ-specific NK cell migration and expansion in the metastatic organ, and to decrease toxicity associated with systemic IL-2.

EXPERIMENTAL DESIGN—Five human osteosarcoma cell lines and 103 patient samples (47 primary and 56 metastatic) were analyzed for NKG2D ligand [NKG2DL] expression. Therapeutic efficacy of aerosol IL-2 + NK cells was evaluated *in vivo* compared with aerosol IL-2 alone and NK cells without aerosol IL-2.

RESULTS—Osteosarcoma cell lines and patient samples expressed various levels of NKG2DL. NK-mediated killing was NKG2DL-dependent and correlated with expression levels. Aerosol IL-2 increased NK cell numbers in the lung and within metastatic nodules but not in other organs. Therapeutic efficacy, as judged by tumor number, size, and quantification of apoptosis, was also increased compared with NK cells or aerosol IL-2 alone. There were no IL-2-associated systemic toxicities.

CONCLUSION—Aerosol IL-2 augmented the efficacy of NK cell therapy against osteosarcoma lung metastasis, without inducing systemic toxicity. Our data suggest that lung-targeted IL-2 delivery circumvents toxicities induced by systemic administration. Combining aerosol IL-2 with NK cell infusions, may be a potential new therapeutic approach for patients with osteosarcoma lung metastasis.

Keywords

osteosarcoma; natural killer cells; aerosol interleukin-2; lung metastasis

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Introduction

The 5-year survival rate of patients with osteosarcoma has not significantly improved in 20 years, remaining at 67% [1]. The lung is the most frequent site of metastasis [2]. Patients who present with pulmonary metastasis at diagnosis, have a 5-year survival rate of 30% [3]. Standard treatment for relapsed patients or those presenting with lung metastasis includes surgical excision followed by chemotherapy. Once metastasis occurs, the prognosis is poor with limited therapeutic options and the current therapies have limited efficacy. The discovery and development of alternative therapeutic approaches is imperative.

We previously demonstrated the efficacy of immune-based therapies against osteosarcoma lung metastasis in both preclinical and clinical settings. We demonstrated that liposomal muramyl tripeptide [L-MTP-PE] activates the tumor-killing properties of blood monocytes [4]. In a Phase II trial, L-MTP-PE significantly increased the disease-free and long-term survival of patients with relapsed osteosarcoma [5]. A Phase III trial demonstrated that the addition of L-MTP-PE to chemotherapy significantly increased the 6-year overall survival in patients with newly diagnosed osteosarcoma [6]. We also demonstrated the effectiveness of genetically modified T cells which recognize IL-11R α on the surface of osteosarcoma cells in a preclinical model [7]. The success of these immunotherapies against osteosarcoma supports the study of other immune-based therapies, such as natural killer [NK] cell therapy.

NK cells are a subset of lymphocytes that lyse tumor cells without prior sensitization [8]. NK cells' recognition of a target cell is a complex interplay between inhibitory and activating NK receptors and their respective ligands [9]. Major activating NK receptors are NKG2D, DNAM and the natural cytotoxicity receptors (NKp46, NKp33, NKp44) [10–15]. NKG2D recognizes MIC A/B [10] and the ULBP proteins [11], which are overexpressed in several cancers [12–14]. Patients with lower peripheral NK activity had increased cancer predisposition [16, 17] and worse prognosis [18]. Additionally the survival of AML and ALL patients treated with T cell-depleted allogeneic hematopoietic transplantation is significantly increased if there is NK alloreactivity between the donor and recipient [19].

Clinical trials involving lymphokine-activated killer [LAK] cells demonstrated minimal clinical benefit [20, 21]. The use of systemic IL-2 did not improve LAK cell efficacy but added significant toxicity [22]. Trials using allogeneic NK cells demonstrated better clinical responses. However, clinical trials were still limited by low NK cell numbers [23, 24]. Using IL-2 and a genetically modified K562 artificial antigen presenting cell [aAPC], Denman *et al* established an *ex vivo* method to expand donor NK cells with increased cytotoxicity to clinically relevant levels [25].

Another obstacle for NK cell therapy is their limited life span *in vivo*. This problem can be circumvented by combining NK cell therapy with IL-2 infusions. Unfortunately, high dose IL-2 induces life-threatening side effects, including oliguria, hypotension, and elevated bilirubin and creatinine levels [22]. Since osteosarcoma metastasizes to the lung, we propose using aerosol IL-2 to increase infused NK cells in the lung selectively. The use of aerosol IL-2 should decrease its systemic effects, in addition to providing organ -specific delivery to expand the number of injected NK cells. We have had success using aerosolized

chemotherapeutic agents to treat osteosarcoma pulmonary metastasis in preclinical mouse models. We demonstrated that aerosol IL-12 gene therapy [26], gemcitabine [27] and 9-nitrocamptothecin [28] induced regression of osteosarcoma lung metastasis. Aerosol IL-2 therapy in dogs with spontaneous pulmonary osteosarcoma metastasis was safe [29]. Clinical trials for pulmonary metastatic renal cell carcinoma using aerosol IL-2 demonstrated minimal toxicity and an improved 5-year survival compared with systemic IL-2 [30].

Here we demonstrate that aerosol IL-2 augments the efficiency of NK cell therapy. Aerosol IL-2 increased organ specific migration and NK cell expansion in the lung, the numbers of NK cells in the individual tumor nodules, and tumor cell killing. Aerosol IL-2 also resulted in decreased systemic exposure, with no evidence of toxicity. Aerosol IL-2 in combination with NK cell therapy may be a novel therapeutic strategy for the treatment of osteosarcoma lung metastasis.

Materials and Methods

Osteosarcoma cell lines and culture

Human osteosarcoma cell lines KRIB, LM7, CCH-OS-D, U2OS, and TE-85 and mouse K7M3 cells were cultured as previously described [27]. All cell lines were mycoplasma-negative and validated by DNA fingerprinting using the AmpFLSTR Identifier kit (Applied Biosystems). The authenticity of cells was determined by the Characterized Cell Line Core at The University of Texas MD Anderson Cancer Center.

Human NK cells: Isolation, *ex vivo* expansion and culture

Human NK Cells were harvested from buffy coats (Gulf Coast Regional Blood Center, Houston, TX) after informed consent [25], and cultured in RPMI medium with 10% fetal bovine serum [FBS], 2 mmol/L glutamine, 1 mmol/L sodium pyruvate and 50 IU/ml recombinant human IL-2 (Proleukin, Novartis, Inc.) Genetically-engineered K562 cells with membrane-bound IL-15 and membrane-bound IL-21 were used as aAPCs after exposure to gamma-irradiation (100-Gy) for *in vitro* expansion of isolated NK cells [25]. Red blood cells were added to enhance agglutination and deplete unwanted cells if further purification was required [31].

Flow cytometry

Phycoerythrin [PE]-conjugated mouse anti-human NKG2D, PE-conjugated mouse anti-human CD16, PE-conjugated mouse anti-human CD3, and allophycocyanin [APC]-conjugated mouse anti-human CD56 (BD Pharmingen) were used to monitor NK phenotype weekly using flow cytometry. Fluorescein isothiocyanate [FITC]-conjugated mouse anti-human HLA-ABC and PE-conjugated mouse anti-human MIC A/B from BD Pharmingen and PE-conjugated mouse anti-human ULBP2/5/6, PE-conjugated mouse anti-human ULBP3 and PE-conjugated mouse anti-human ULBP1 from R&D Systems, were used to determine HLA and NKG2D ligand [NKG2DL] expression on human osteosarcoma cells. Cells were suspended in phosphate-buffered saline [PBS] containing 2% FBS and incubated with the indicated antibodies for 20 minutes at 4°C. Data were acquired using a

FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). APC- and PE-conjugated isotype-control IgG antibodies were used as negative controls. Human NK cells were defined as CD56⁺, CD16⁺, NKG2D⁺ and CD3⁻. NK cell purity of at least 95% was required for further use.

Cytotoxicity assays

NK-mediated-cytotoxicity against osteosarcoma cells following a 4-hour co-incubation period was measured using a [³H]thymidine incorporation assay [32].

To determine the importance of NKG2D-ligand interaction, cytotoxicity assays were performed where NKG2D or a NKG2DL was blocked. Osteosarcoma cells were plated in triplicate on 96-well plates, labeled with [³H] thymidine for 24 hours at 37°C, washed with PBS and incubated with mouse anti-human ULBP2/5/6 (R&D Systems) for 24 hours at 37°C. The cells were then washed with PBS co-cultured with NK cells for 4 hours at 37°C, and incubated with mouse anti-human NKG2D (R&D Systems). Prior to co-culture with osteosarcoma cells, NK cells were incubated with mouse anti-human NKG2D for 1 hour at 37°C. Cytotoxicity was quantified as described previously [32].

Patient osteosarcoma samples

Microarray slides from paraffin-embedded osteosarcoma tumor specimens contained 47 primary osteosarcoma samples and 56 osteosarcoma pulmonary metastasis samples. The institutional review board approved medical record reviews for the current study.

NKG2DL expression was determined using recombinant human NKG2D/Fc chimera (R&D Systems) and immunohistochemical staining [33]. Sections not exposed to recombinant human NKG2D/Fc served as negative controls. The expression of the NKG2DL ULBP2 is high in terminally differentiated normal human cervix epithelium (US Biomax Inc.) which served as the positive control [34].

Animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center. Four-week-old *nu/nu* and *BALB/C* mice were purchased from the National Cancer Institute (Bethesda, MD).

Aerosol treatment was performed as described previously [27, 28]. PBS suspension (10 mL) with or without recombinant human IL-2 [IL-2] (TECIN™ Teceleukin, Bulk Ro 23-6019, National Cancer Institute) was added to an AeroTech II nebulizer (CIS-USA). Mice were exposed to the aerosol for 1 hour.

To determine human donor NK cell retention in mouse lungs without metastasis, *nu/nu* mice were treated with aerosol IL-2 @ 2000 U or PBS. We injected 50 million CM-DiI (Molecular Probes)-labeled human NK cells/mouse intravenously through the tail vein. Aerosol treatment was administered 24 hours prior to NK cell injection, the day of NK cell injection, and then every other day for 1 week. Mice were killed 1 day, 3 days or 1 week after NK injection. The lungs, spleen, liver, heart and kidneys of the mice were removed,

embedded in Tissue-Tek optimum cutting temperature compound [OCT] (Fischer Scientific), and frozen. Lungs were expanded with OCT: PBS at a 1:1 dilution.

Toxicity of aerosol IL-2 was determined using immunocompetent mice. BALB/C mice were treated with either aerosol IL-2 @ 2000 U or PBS every other day for 1 week. Mice were killed 3 days and 1 week after the start of treatment and 1 month after the treatment ended. The lungs, spleen, liver, heart and kidney of the mice were removed fixed in formalin, and embedded in paraffin.

To determine the therapeutic effect of aerosol IL-2 + NK cells, 3 million LM7 cells/mouse were injected i.v. through the tail vein of *nu/nu* mice. The presence of micrometastasis was confirmed at 5 weeks. Treatment was then initiated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells 6 weeks after LM7 injection. Aerosol therapy continued every other day for 5 weeks. NK cell injections (5×10^7 cells/mouse) were given 2 times a week starting 1 day after the first aerosol treatment. Mice were sacrificed 5 weeks after start of treatment. Lungs were extracted, expanded with OCT:PBS at a 1:1 dilution, embedded in OCT and frozen. Spleen, liver, heart and kidneys were removed, fixed in formalin and embedded in paraffin. This experiment was repeated twice to confirm results.

To assess aerosol IL-2 toxicity, complete blood count [CBC] and liver enzyme blood chemistry were analyzed in mice treated with aerosol PBS or aerosol IL-2. Serum IL-2 concentrations were determined using a Human IL-2 High Sensitivity ELISA kit (eBioscience), with absorbance measured using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices).

Immunofluorescence

To determine the presence of CM-DiI-labeled human NK cells in mouse organs following therapy, frozen sections of lung, spleen, liver, kidney and heart were fixed in acetone and stained with Hoechst33342 nucleic acid stain (Molecular Probes) at a 1:10,000 dilution in PBS. The corresponding organs of mice not treated with CM-Dil-labeled human NK Cells were used as the controls.

Frozen sections of lung with LM7 osteosarcoma metastasis were examined for the presence of NK cells using anti-human NKG2D antibody. These sections were incubated with 5% horse serum and 1% goat serum in PBS for 30 minutes, then incubated overnight 4°C with AffiniPure Fab Fragment goat anti-mouse IgG (Jackson ImmunoResearch, Inc.). Sections were then incubated overnight at 4°C with mouse anti-human NKG2D (eBioscience), followed by incubation with goat anti-mouse Alexa Fluor® 546 (Molecular Probes) at RT for 1 hour and then with Hoechst33342 (Molecular Probes) for ten minutes. Negative controls were made by omission of the primary antibody. Mean fluorescence per field was quantified in 5 random fields for each section by using the Simple PCI software (Hamamatsu, Inc.).

TUNEL staining

Frozen sections of tumor were fixed with 4% paraformaldehyde and incubated with Proteinase K (Promega DeadEnd Fluorometric TUNEL System) for 10 minutes, incubated

with 3% H₂O₂ for 12 minutes to block endogenous peroxidase, and then incubated overnight at 4°C with recombinant terminal transferase (Promega DeadEnd Fluorometric TUNEL System) and biotin-16-dUTP (Roche Applied Sciences). Sections were then treated with 2% bovine serum albumin plus + 5% normal horse serum in distilled water for 10 minutes, followed by a 30-minute incubation with 4+Streptavidin-HRP-Label (Biocare Medical). This was followed by a DAB 10 minute incubation and light counterstain with hematoxylin. Mean positive TUNEL staining was quantified by the Simple PCI software (Hamamatsu, Inc.) in 5 random fields for each section.

Statistical analysis

The unpaired Student's t-test was used to evaluate the significance of differences between experimental groups. The inverse correlation between apoptosis and tumor number was evaluated using Spearman's rank correlation test and a linear regression analysis. $P < 0.05$ was considered significant.

Results

Human osteosarcoma cells are susceptible to human NK cytotoxicity

To determine the potential susceptibility of osteosarcoma to NK cells, the expression of NKG2D ligands on 5 different human osteosarcoma cell lines was determined (Figure 1A). With the exception of KRIB, all the cell lines expressed a high percentage of HLA-A,B,C and ULBP2. In addition, MIC A/B was expressed on LM7, U2OS and TE-85, while ULBP1 was expressed in CCH-OS-D. LM7 [35], U2OS [36], CCH-OS-D [37] and TE-85 [36] are non-transformed cell lines, whereas KRIB was derived from HOS transected with v-K-ras [36]. This may explain the difference between KRIB and the 4 other cell lines.

NK cytotoxicity against LM7, CCH-OS-D and KRIB cells was determined. Since NK-mediated cytotoxicity is species-specific, K7M3 mouse osteosarcoma cells were used as the negative control. NK cells were cytotoxic to all 3 human cell lines. KRIB cells were less sensitive (Figure 1B). At a 1:1 effector: target [E:T] ratio, the NK-mediated cytotoxicity against LM7 and CCH-OS-D cells was 45% compared to 10%. The low level of NKG2D ligands on KRIB cells correlated with the low cytotoxicity value.

NK-mediated cytotoxicity is dependent on the interaction between the NKG2D receptor on NK cells and its corresponding ligand on the tumor cell. Blocking the NKG2D receptor with antibody significantly reduced NK cytotoxicity. At a 1:5 E:T ratio, 10 µg/mL of anti-NKG2D antibody decreased NK-mediated cytotoxicity from $70\% \pm 15$ to $22.6\% \pm 10.8$ ($P = 0.02$). At 1:10 E:T ratio cytotoxicity decreased from $73\% \pm 6.2$ to $12\% \pm 12.7$ ($P = 0.02$). Similarly blocking the ligand also decreased NK-mediated killing. ULBP2 was selected since this ligand is expressed on all the non-transformed cell lines. Blocking ULBP2 in LM7 significantly reduced NK mediated killing. At a 1:5 E:T ratio, 10 µg/mL of anti-ULBP2 antibody decreased cytotoxicity from $70\% \pm 15$ to $29.8\% \pm 10$ ($P = 0.004$). At a 1:10 E:T ratio, cytotoxicity decreased from $73\% \pm 6.2$ to $39\% \pm 9.4$ ($P = 0.01$).

The expression of NKG2D ligands in osteosarcoma patient samples

To determine whether NKG2D ligands were expressed in osteosarcoma patient tumors, a tissue microarray with 47 primary osteosarcoma and 56 osteosarcoma lung metastasis samples was evaluated by immunohistochemistry. NKG2D ligands were expressed in 27(57%) of the 47 primary tumor specimens. Staining was weak in 18 specimens (38%), moderate in 6 specimens (12.8%) and strong in 3 specimens (6.4%) (Supplementary Figure 1A). NKG2D ligands were expressed in 44 (77%) of the 56 lung metastasis specimens. Staining was weak in 22 specimens (39%), moderate in 18 specimens (32%) and strong in 4 specimens (7.5%) (Supplementary Figure 1B).

Aerosol IL-2 increased NK cells in the lungs, but not in the heart, liver, kidney or spleen

To determine whether aerosol IL-2 increased the presence of infused NK cells in the lung, two groups of mice were injected i.v. with 5×10^7 CM-DiI-labeled human NK cells/mouse. The mice were treated with either aerosol IL-2 or aerosol PBS. Aerosol IL-2 increased the number of NK cells in the lung 1 and 3 days after NK cell injection (Figure 2A). This effect was not seen on day 7. By contrast, there was no difference in the number of human NK cells in the liver, spleen, heart and kidney in the mice treated with aerosol IL-2 compared to the mice treated with aerosol PBS (Figure 2B,C).

Aerosol IL-2 increased the efficacy of NK cells *in vivo*

LM7 cells were injected i.v. into nude mice. The presence of micro-metastasis was confirmed at 5 weeks. Treatment was initiated 1 week later. Therapy was given twice a week for 5 weeks. Aerosol IL-2 + NK cell therapy significantly reduced the number and the size of the metastasis when compared to aerosol PBS, aerosol IL-2, and aerosol PBS + NK cells ($P = 0.01$; Figure 3A–C). Four of 9 mice treated with aerosol IL-2 + NK therapy had no visible metastasis (Figure 3A). Aerosol IL-2 + NK cell therapy also significantly reduced the total area of the lungs covered in metastases (Figure 3D) compared to that of mice treated with aerosol PBS ($P = 0.004$), aerosol IL-2 ($P = 0.04$) or aerosol PBS + NK cells ($P = 0.04$).

Having demonstrated increased NK cells in the lungs and decreased tumor burden following aerosol IL-2, we wished to determine whether there were increased NK cells associated with the tumor nodules. Fluorescent microscopy demonstrated increased NKG2D staining in the lung metastasis from the mice treated with aerosol IL-2 compared to that from the mice treated with aerosol PBS ($P = 0.038$; Figure 4).

TUNEL staining was used to evaluate apoptosis in the tumor nodules (Figure 4A). Apoptosis was significantly higher in lung metastases from the mice treated with aerosol IL-2 + NK cells than that from the mice treated with aerosol PBS ($P = 0.009$), aerosol IL-2 ($P = 0.02$) or aerosol PBS + NK cells ($P = 0.05$). The level of apoptosis inversely correlated with tumor burden, as quantified by the number of metastases ($R = -0.788$, $P = 0.008$), tumor diameter ($R = -0.89$, $P = 0.008$) and metastatic area ($R = -0.78$, $P = 0.0009$) using the Spearman rank correlation test. This inverse correlation was validated by a linear regression analysis (Figure 4B).

Toxicity studies were performed on both nude and immunocompetent BALB/C mice. Histological examination of the spleen, lung, liver, heart and kidney of nude mice treated for 1 week and 1 month with aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells showed no signs of acute inflammation, scarring, or toxicity, and the results did not differ from those for mice treated with aerosol PBS (Supplementary Figure 2A,B). There were no abnormalities in the CBC and liver enzymes. We also saw no acute or chronic inflammation, scarring, or other signs of toxicity in the organs from BALB/C mice treated with aerosol IL-2 for 1 week and 1 month. BALB/C mice were not treated with human NK cells as they have an intact immune system that will reject these cells.

Serum IL-2 levels following aerosol IL-2

Serum IL-2 levels were measured in mice treated with aerosol IL-2 twice weekly for 2 and 5 weeks. Aerosol PBS treatment was used as the negative control. The positive control was serum from mice treated with 20,000U IL-2 i.p, the dose usually used in therapeutic studies evaluating cellular therapy [38]. Aerosol IL-2 given for 2 weeks did not significantly increase serum IL-2 levels compared aerosol PBS ($P = 0.3$). While aerosol IL-2 given for 5 weeks resulted in mild elevations in serum IL-2 ($P=0.012$), these levels were significantly lower than the IL-2 levels measured in mice treated with a single dose of 20,000 U IL-2 i.p. (Figure 6).

Discussion

With the exception of L-MTP-PE, which increased the long-term survival when used in combination with chemotherapy [6], there have been no new drugs developed for either newly diagnosed or relapsed osteosarcoma. Salvage chemotherapy has made no impact on long-term survival [39]. The success of L-MTP-PE immunotherapy in decreasing the mortality rate by 30% [6], paves the way for the development of other types of immunotherapy that can target osteosarcoma lung metastases.

NK cells are a component of the innate immune system that recognize and kill malignant and virally infected cells but not normal host cells. The data presented here indicate that NK cells in combination with aerosol IL-2 have therapeutic potential against osteosarcoma lung metastases. NKG2D ligands, which are required for NK-mediated cytotoxicity, were expressed by human osteosarcoma cells and in patient tumor specimens from both the primary tumors and lung metastases. There was a higher percentage of cells expressing NKG2DL in the pulmonary metastases (77%) than in the primary tumors (57%), further validating the potential of NK cell therapy for relapsed disease in the lungs. Expression of NKG2DL correlated with the magnitude of killing *in vitro*. This was best demonstrated by the KRIB cells, where the expression of ULBP 1/2/3 is < 15%. NK-mediated cytotoxicity was <20 % even at an E:T ratio of 20:1. Conversely, NK-mediated cytotoxicity was significantly decreased by blocking either the NKG2D receptor on NK cells or its ligand on the tumor cells.

We evaluated the *in vivo* activity of NK cells alone and in combination with aerosol IL-2. While the intravenous injection of fluorescently-labeled NK cells resulted in localization in the lung, the use of aerosol IL-1 significantly increased the number of cells in the lung at 1

and 3 days post injection. This localization in the lung was organ specific as labeled NK cells were not found in the liver, heart, or kidney. Labeled NK cells were observed in the spleen, but there was no difference between mice treated with aerosol PBS and aerosol IL-2. We further demonstrated that mice treated with aerosol IL-2 had increased numbers of NK cells within the pulmonary tumor nodules. This is an important finding as the level of NK-mediated cytotoxicity depends upon the E:T ratio. Having increased numbers of NK cells in the tumor should translate into increased efficacy. In our therapeutic *in vivo* studies, mice who received aerosol IL-2 + NK cells for 5 weeks had significantly fewer lung metastases than mice who received aerosol PBS alone, NK cells alone, or NK cells in combination with aerosol PBS. The metastases in the mice treated with aerosol IL-2 + NK cells were also smaller with increased apoptosis compared to the other 3 groups. The level of apoptosis was inversely correlated with both tumor numbers and tumor size. These data confirm that aerosol IL-2 increased not only the number of NK cells in the lung and the NK cell content within the tumor nodule but also, the level of tumor cell apoptosis and the therapeutic success of NK cell therapy.

NK cell killing is mediated by the release of cytotoxic granules that trigger the apoptotic pathway [40]. A higher E:T ratio results in increased tumor cell apoptosis. Our data suggests that the reduction in the number and size of the tumor nodules was a direct effect of the increased number of NK cells within the tumor nodule. IL-2 has been shown to increase the surface expression of NK cell receptors. Therefore, the increased therapeutic activity seen with aerosol IL-2 may also reflect a more efficient killing process mediated by increased receptors on NK cells.

IL-2 combined with cell therapy is required to sustain the activation and viability of injected NK cells [41, 42]. Unfortunately, systemic IL-2 is associated with severe toxicity including oliguria, hypotension, elevated liver enzymes, and edema which limits its use [22]. Our rationale for using aerosol IL-2 was to induce selective migration and expansion of NK cells in the lung, the organ where osteosarcoma metastasizes almost exclusively. The use of aerosol IL-2 should result in a higher concentration in the target organ and a lower systemic concentration resulting in fewer side effects. The dose used in our own studies is 1/10 the dose used when IL-2 is administered systemically to support immune cell therapy. Histologic examination of the spleen, lung, liver, kidney, and heart from mice treated with aerosol IL-2 for 1 week and 1 month showed no evidence of acute or chronic inflammation, scarring, edema, or other organ damage. Serum liver enzymes and CBC were within normal limits. Serum IL-2 levels following aerosol IL-2 were well below those found in mice treated with intraperitoneal IL-2. Therefore, aerosol IL-2 is not only effective in augmenting the activity of NK cell therapy but can be given at a lower dose than required systemically and is an alternative to systemic IL-2 for use with NK cell therapy against lung metastases.

The safety and tolerance of using aerosol IL-2 has been well documented in cancer and immune-deficient patients [43–45]. Aerosol IL-2 resulted in a dose-dependent expansion of activated lymphocytes with increased HLA-DR expression in the broncho-alveolar lavage fluid when compared with lavage fluid obtained before treatment [43]. The CD4:CD8 ratio did not change indicating that suppressor T cells were probably not induced. The lymphocytes stimulated by aerosol IL-2 were predominantly of a memory cell phenotype.

Similar to what we observed in our studies, there was no significant change in the number or phenotype of lymphocytes in the peripheral blood. By contrast, systemic IL-2 caused an alteration in both the number and phenotype of peripheral blood lymphocytes [46, 47]. Peak serum concentrations following aerosol IL-2 given at $2 \times 10^5 - 1.2 \times 10^6$ IU were 1% of those reported after intravenous injection [48]. Furthermore, quality of life analysis in patients receiving aerosol IL-2 was higher than in patients treated with systemic IL-2 [44]. Aerosol IL-2 was safe when given to dogs [49]. Similar to the results cited above, there was an increase in bronchoalveolar lavage lymphocyte counts but no change in the CD4:CD8 ratio in dogs treated with aerosol IL-2 [49].

Our data show that the aerosol IL-2 is an effective way to induce selective migration and expansion of NK cells in the lungs. Aerosol IL-2 increased NK cell numbers in the lung tumor nodules, and increased tumor cell apoptosis. Combining aerosol IL-2 with NK cells increased the efficacy of NK cell therapy without causing systemic toxicity. Since the safety and tolerability of aerosol IL-2 have already been documented in several clinical trials, this organ-specific cytokine delivery concept can be exploited to target NK cell migration, expansion, and activation selectively in the lung. This combination therapy may therefore be a new therapeutic approach for patients with relapsed, unresponsive osteosarcoma lung metastases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

OS	osteosarcoma
NK	natural killer
LAK	lymphokine activated killer
aAPC	artificial antigen presenting cell
CBC	complete blood count

References

1. Smith MA, Seibel NL, Altekruse SF, Ries LA, Melbert DL, O'Leary M, et al. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. *J Clin Oncol.* 2010; 28(15): 2625–2634. [PubMed: 20404250]
2. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on

- neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol.* 2002; 20(3):776–790. [PubMed: 11821461]
3. Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. *J Clin Oncol.* 2003; 21(10):2011–2018. [PubMed: 12743156]
 4. Kleinerman ES, Erickson KL, Schroit AJ, Fogler WE, Fidler IJ. Activation of tumoricidal properties in human blood monocytes by liposomes containing lipophilic muramyl tripeptide. *Cancer Res.* 1983; 43(5):2010–2014. [PubMed: 6831430]
 5. Kleinerman ES, Gano JB, Johnston DA, Benjamin RS, Jaffe N. Efficacy of liposomal muramyl tripeptide (CGP 19835A) in the treatment of relapsed osteosarcoma. *Am J Clin Oncol.* 1995; 18(2): 93–99. [PubMed: 7900714]
 6. Meyers PA, Schwartz CL, Krailo MD, Healey JH, Bernstein ML, Betcher D, et al. Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival--a report from the Children's Oncology Group. *J Clin Oncol.* 2008; 26(4):633–638. [PubMed: 18235123]
 7. Huang G, Yu L, Cooper LJ, Hollomon M, Huls H, Kleinerman ES. Genetically modified T cells targeting interleukin-11 receptor alpha-chain kill human osteosarcoma cells and induce the regression of established osteosarcoma lung metastases. *Cancer Res.* 2012; 72(1):271–281. [PubMed: 22075555]
 8. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol.* 1975; 5(2):117–121. [PubMed: 1086218]
 9. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunol Rev.* 2008; 224:70–84. [PubMed: 18759921]
 10. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science.* 1999; 285(5428):727–729. [PubMed: 10426993]
 11. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity.* 2001; 14(2):123–133. [PubMed: 11239445]
 12. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A.* 1999; 96(12):6879–6884. [PubMed: 10359807]
 13. Heinemann A, Paschen A. Tumor suppressors control ULBP2, an innate surface ligand of the lymphocyte immune receptor NKG2D. *Oncoimmunology.* 2012; 1(4):535–536. [PubMed: 22754777]
 14. de Kruijf EM, Sajet A, van Nes JG, Putter H, Smit VT, Eagle RA, et al. NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: an observational study. *BMC Cancer.* 2012; 12:24. [PubMed: 22257486]
 15. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med.* 2003; 198(4):557–567. [PubMed: 12913096]
 16. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet.* 2000; 356(9244):1795–1799. [PubMed: 11117911]
 17. Furue H, Matsuo K, Kumimoto H, Hiraki A, Suzuki T, Yatabe Y, et al. Decreased risk of colorectal cancer with the high natural killer cell activity NKG2D genotype in Japanese. *Carcinogenesis.* 2008; 29(2):316–320. [PubMed: 18174257]
 18. Mamessier E, Pradel LC, Thibault ML, Drevet C, Zouine A, Jacquemier J, et al. Peripheral blood NK cells from breast cancer patients are tumor-induced composite subsets. *J Immunol.* 2013; 190(5):2424–2436. [PubMed: 23359508]
 19. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* 2002; 295(5562):2097–2100. [PubMed: 11896281]

20. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med.* 1982; 155(6):1823–1841. [PubMed: 6176669]
21. Phillips JH, Gemlo BT, Myers WW, Rayner AA, Lanier LL. In vivo and in vitro activation of natural killer cells in advanced cancer patients undergoing combined recombinant interleukin-2 and LAK cell therapy. *J Clin Oncol.* 1987; 5(12):1933–1941. [PubMed: 3500280]
22. Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med.* 1987; 316(15):889–897. [PubMed: 3493432]
23. Iliopoulou EG, Kountourakis P, Karamouzis MV, Doufexis D, Ardavanis A, Baxevanis CN, et al. A phase I trial of adoptive transfer of allogeneic natural killer cells in patients with advanced non-small cell lung cancer. *Cancer Immunol Immunother.* 2010; 59(12):1781–1789. [PubMed: 20703455]
24. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J Clin Oncol.* 2010; 28(6):955–959. [PubMed: 20085940]
25. Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One.* 2012; 7(1):e30264. [PubMed: 22279576]
26. Jia SF, Worth LL, Densmore CL, Xu B, Duan X, Kleiner ES. Aerosol gene therapy with PEI: IL-12 eradicates osteosarcoma lung metastases. *Clin Cancer Res.* 2003; 9(9):3462–3468. [PubMed: 12960138]
27. Koshkina NV, Kleiner ES. Aerosol gemcitabine inhibits the growth of primary osteosarcoma and osteosarcoma lung metastases. *Int J Cancer.* 2005; 116(3):458–463. [PubMed: 15800950]
28. Knight V, Kleiner ES, Waldrep JC, Giovanella BC, Gilbert BE, Koshkina NV. 9-Nitrocamptothecin liposome aerosol treatment of human cancer subcutaneous xenografts and pulmonary cancer metastases in mice. *Ann N Y Acad Sci.* 2000; 922:151–163. [PubMed: 11193890]
29. Khanna C, Anderson PM, Hasz DE, Katsanis E, Neville M, Klausner JS. Interleukin-2 liposome inhalation therapy is safe and effective for dogs with spontaneous pulmonary metastases. *Cancer.* 1997; 79(7):1409–1421. [PubMed: 9083164]
30. Huland E, Heinzer H, Jorres RA, Loppow D, Huland H. Therapeutic approaches in metastatic renal cell carcinoma: local immunotherapy. *Urologe A.* 2004; 43(Suppl 3):S140–S144. [PubMed: 15179553]
31. Warren HS, Rana PM. An economical adaptation of the RosetteSep procedure for NK cell enrichment from whole blood, and its use with liquid nitrogen stored peripheral blood mononuclear cells. *J Immunol Methods.* 2003; 280(1–2):135–138. [PubMed: 12972194]
32. Jia SF, Zwelling LA, McWatters A, An T, Kleiner ES. Interleukin-1 alpha increases the cytotoxic activity of etoposide against human osteosarcoma cells. *J Exp Ther Oncol.* 2002; 2(1):27–36. [PubMed: 12415617]
33. Gordon N, Arndt CA, Hawkins DS, Doherty DK, Inwards CY, Munsell MF, et al. Fas expression in lung metastasis from osteosarcoma patients. *J Pediatr Hematol Oncol.* 2005; 27(11):611–615. [PubMed: 16282894]
34. Textor S, Durst M, Jansen L, Accardi R, Tommasino M, Trunk MJ, et al. Activating NK cell receptor ligands are differentially expressed during progression to cervical cancer. *Int J Cancer.* 2008; 123(10):2343–2353. [PubMed: 18712710]
35. Jia SF, Worth LL, Kleiner ES. A nude mouse model of human osteosarcoma lung metastases for evaluating new therapeutic strategies. *Clin Exp Metastasis.* 1999; 17(6):501–506. [PubMed: 10763916]
36. Dass CR, Ek ET, Choong PF. Human xenograft osteosarcoma models with spontaneous metastasis in mice: clinical relevance and applicability for drug testing. *J Cancer Res Clin Oncol.* 2007; 133(3):193–198. [PubMed: 17031670]

37. Geryk-Hall M, Yang Y, Hughes DP. Driven to death: inhibition of farnesylation increases Ras activity in osteosarcoma and promotes growth arrest and cell death. *Mol Cancer Ther.* 2010; 9(5): 1111–1119. [PubMed: 20406948]
38. Shimizu K, Fields RC, Giedlin M, Mule JJ. Systemic administration of interleukin 2 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines. *Proc Natl Acad Sci U S A.* 1999; 96(5):2268–2273. [PubMed: 10051630]
39. Leary SE, Wozniak AW, Billups CA, Wu J, McPherson V, Neel MD, et al. Survival of pediatric patients after relapsed osteosarcoma: The St. Jude Children's Research Hospital experience. *Cancer.* 2013
40. Lugini L, Cecchetti S, Huber V, Luciani F, Macchia G, Spadaro F, et al. Immune surveillance properties of human NK cell-derived exosomes. *J Immunol.* 2012; 189(6):2833–2842. [PubMed: 22904309]
41. Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, et al. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *J Clin Invest.* 1993; 91(1):123–132. [PubMed: 7678599]
42. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, et al. Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients with Metastatic Cancer. *New England Journal of Medicine.* 1985; 313(23):1485–1492. [PubMed: 3903508]
43. Lorenz J, Wilhelm K, Kessler M, Peschel C, Schwulera U, Lissner R, et al. Phase I trial of inhaled natural interleukin 2 for treatment of pulmonary malignancy: toxicity, pharmacokinetics, and biological effects. *Clin Cancer Res.* 1996; 2(7):1115–1122. [PubMed: 9816276]
44. Heinzer H, Mir TS, Huland E, Huland H. Subjective and objective prospective, long-term analysis of quality of life during inhaled interleukin-2 immunotherapy. *J Clin Oncol.* 1999; 17(11):3612–3620. [PubMed: 10550161]
45. Huland E, Heinzer H. Renal cell carcinoma - innovative medical treatments. *Curr Opin Urol.* 2004; 14(4):239–244. [PubMed: 15205581]
46. Hermann GG, Geertsens PF, von der Maase H, Zeuthen J. Interleukin-2 dose, blood monocyte and CD25+ lymphocyte counts as predictors of clinical response to interleukin-2 therapy in patients with renal cell carcinoma. *Cancer Immunol Immunother.* 1991; 34(2):111–114. [PubMed: 1760814]
47. Salvo G, Samoggia P, Masciulli R, Boccoli G, Allavena P, Mariani G, et al. Interleukin-2 bolus therapy induces immediate and selective disappearance from peripheral blood of all lymphocyte subpopulations displaying natural killer activity: role of cell adhesion to endothelium. *Eur J Cancer.* 1992; 28A(4–5):818–825. [PubMed: 1524901]
48. Aulitzky WE, Schuler M, Peschel C, Huber C. Interleukins. Clinical pharmacology and therapeutic use. *Drugs.* 1994; 48(5):667–677. [PubMed: 7530625]
49. Khanna C, Hasz DE, Klausner JS, Anderson PM. Aerosol delivery of interleukin 2 liposomes is nontoxic and biologically effective: canine studies. *Clin Cancer Res.* 1996; 2(4):721–734. [PubMed: 9816223]

Translational Relevance

Little progress has been made in the treatment of osteosarcoma lung metastasis. We show that NK cell therapy combined with aerosol interleukin-2 [IL-2] eradicated osteosarcoma metastasis and increased survival. Osteosarcoma cells and patient tumor samples expressed NK cell ligands. Aerosol IL-2 resulted in selective migration and retention of NK cells in the lung but not in the liver, spleen, kidney, or heart as well as an increased number of NK cells in the tumor nodules. Complete blood counts and liver enzyme levels were normal following therapy. No systemic toxicity as determined by organ histology was seen following 5 weeks of treatment with aerosol IL-2 in either immunocompromised or immunocompetent mice. These data indicate that combination aerosol IL-2 + NK cell therapy may be a novel therapeutic approach for the treatment of osteosarcoma lung metastasis.

A

B

Figure 1. Human osteosarcoma cells express NKG2D ligands

A. Human osteosarcoma cells were analyzed for expression of NKG2D ligands and HLA-ABC using flow cytometry. **B.** NK-cell-mediated cytotoxicity was quantified using [^3H]-thymidine cytotoxicity assay. K7M3 mouse osteosarcoma cells were the negative control.

Figure 2. Aerosol IL-2 increases human NK cells in the lungs, but not in the heart, liver, kidney, or spleen

Nude mice were injected with 5×10^7 CM-Dil-labeled human NK cells per mouse. Aerosol PBS or IL-2 was given 1 day prior to and on the day of NK cell infusion and then continued every other day for 1 week. **A.** Fluorescent microscopy was used to detect the presence of CM-Dil+ cells (red) in lungs at 1, 3 and 7 days after NK cell infusion. Cellular nuclei were identified with Hoechst33258 (blue). Mean positive fluorescence was quantified using the Simple PCI software in 5 random fields per section. **B.** Mean positive fluorescence was

quantified for the presence of CM-DiI+ cells in spleen, liver, kidney, and heart. **C.** Representative sections from organs 3 days after NK cell infusion.

A

C

D

Figure 3. Aerosol IL-2 + NK cell therapy inhibits osteosarcoma lung metastasis

Nude mice were injected i.v. with 3×10^6 LM7 cells. Therapy was initiated on week 6. Mice were treated with aerosol PBS, aerosol IL-2, aerosol PBS + NK cells or aerosol IL-2 + NK cells twice weekly for 5 weeks. **A.** Mice were killed and lungs analyzed for visible metastasis. Representative pictures of the lungs **B.** Mean number of lung nodules. **C.** Mean diameter of lung nodules. **D.** For each lung, the total metastatic area was calculated. The mean metastatic area for each group was determined. $P < 0.05$ was considered significant.

Figure 4. Aerosol IL-2 increased NK cells in lung

Fluorescent microscopy was used to determine the NK cell infiltration in the lung using anti-human NKG2D (red). Cellular nuclei were identified with Hoechst33258 (blue). Mean positive fluorescence was quantified using the Simple PCI software in 5 random fields per section. $P < 0.05$ was considered significant.

Figure 5. Aerosol IL-2 + NK cell therapy increased tumor apoptosis

A. Mean positive TUNEL was quantified using the Simple PCI software in 5 random fields per section. **B.** TUNEL Simple PCI quantified values for each lung were correlated to the corresponding number of metastatic nodules, mean diameter of nodules, and total metastatic area. $P < 0.05$ was considered significant.

Figure 6. Serum IL-2 levels after aerosol IL-2 treatment

ELISA was used to determine serum IL-2 for mice treated with aerosol PBS, aerosol IL-2 for 2 weeks, aerosol IL-2 for 5 weeks and 1 dose of 20,000U IL-2 i.p. $P < 0.05$ was considered significant.