Natural Killer Cell Therapy and Aerosol Interleukin-2 for the Treatment of Osteosarcoma Lung Metastasis

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Background. 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.

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-11Rα on 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 target cells 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 [10–15]. NKG2D recognizes MICA/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 patients treated with T cell-depleted allogeneic hematopoietic transplantation is significantly increased if there is NK alloreactivity between donor and recipient [19].

NK cells harvested from donor blood leukapheresis have limited viability in vivo. In order to maintain viability and facilitate treatment, several methods have been investigated to effectively expand and activate NK cells for later patient infusion. Using interleukin-2 (IL-2) and a genetically modified K562 artificial antigen presenting cell (aAPC), Denman et al. [20] established an ex vivo method to expand donor NK cells with increased cytotoxicity and decreased senescence.

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 edema [21]. Since osteosarcoma metastasizes to the lung, we propose using aerosol IL-2 to increase infused NK cells in the lung selectively. This 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 [22], gemcitabine [23] and 9-nitrocamptothecin [24] induced regression of osteosarcoma lung metastasis. Aerosol IL-2 therapy in dogs with pulmonary osteosarcoma metastasis was safe [25]. Clinical trials for pulmonary metastatic renal cell
carcinoma using aerosol IL-2 demonstrated minimal toxicity and improved 5-year survival compared with systemic IL-2 [26].

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 without 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

Osteosarcoma cell lines KRB, LM7, CCH-OS-D, U2OS, TE-85, and K7M3 were cultured as previously described [23]. Cell lines were mycoplasma-negative and were validated by DNA fingerprinting using AmpFLSTR Identifier kit (Applied Biosystems, Carlsbad, CA). Authenticity 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 [20], and cultured in RPMI medium with 10% FBS, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 50 IU/ml recombinant human IL-2 (Proleukin, Novartis, Inc., Basel, CH) Genetically engineered K562 cells with membrane-bound IL-15 and membrane-bound IL-21 were used as aAPCs after gamma-irradiation (100 Gy) for in vitro expansion of isolated NK cells [20].

Flow Cytometry

Phycoerythrin (PE)-conjugated mouse antibodies against human NKG2D, CD16, and CD3 and allophycocyanin (APC)-conjugated mouse anti-human CD56 (BD Pharmingen, San Jose, CA) were used to weekly monitor NK phenotype. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human HLA-ABC and PE-conjugated mouse anti-human MICA/B from BD Pharmingen and PE-conjugated mouse antibodies against human ULBP2/5/6, ULBP3, and ULBP1 from R&D Systems (Minneapolis, MN), were used to determine HLA and NKG2D ligand (NKG2DL) expression. Data were acquired using a FACScalibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Human NK cells were defined as CD56⁺CD16⁻NKG2D⁺CD3⁻. NK cell purity of ≥95% was required for further use.

Cytotoxicity Assays

NK-mediated-cytotoxicity against osteosarcoma cells following a 4-hour co-incubation period was measured using [³H]thymidine incorporation assay [27]. To determine the importance of NKG2D-ligand interaction, cytotoxicity assays were performed where NKG2D or NKG2DL was blocked. Osteosarcoma cells were plated in triplicate on 96-well plates, labeled overnight with [³H] thymidine, washed and incubated overnight with mouse anti-human ULBP2/5/6 (R&D Systems). The cells were washed, co-cultured with NK cells for 4 hours, and incubated with mouse anti-human NKG2D (R&D Systems).

Patient Osteosarcoma Samples

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

NKG2DL expression was determined by immunohistochemical staining with recombinant human NKG2DL/Fc chimera (R&D Systems) [28]. Sections not exposed to NKG2DL/Fc served as negative controls. ULBP2 expression is high in differentiated normal human cervix epithelium (US Biomax, Inc., Rockville, MD) which served as positive control [29].

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 Natural Cancer Institute (Bethesda, MD). Aerosol treatment was performed as described previously [23, 24]. 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 AeroTechII nebulizer (CIS-USA, Bedford, MA). Mice were exposed to 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 at 2,000 U or PBS. We injected 50 million CM-DiI (Molecular Probes, Eugene, OR)-labeled ex vivo expanded human NK cells/mouse intravenously through the tail vein. Aerosol was administered 24 hours prior to NK cell injection, the day of injection, and every other day for 1 week. Mice were killed 1, 3, or 7 days after injection. The lungs, spleen, liver, heart, and kidneys were embedded in Tissue-Tek optimum cutting temperature compound (OCT) (Fischer Scientific, Hampton, NH) and frozen. Lungs were expanded with OCT/PBS at 1:1 dilution.

Toxicity of aerosol IL-2 was determined in immunocompetent BALB/C mice. Mice were treated with aerosol IL-2 at 2,000 U or PBS every other day for 1 week. Mice were killed 3 and 7 days after starting treatment and 1 month after treatment ended. The lungs, spleen, liver, heart, and kidneys were formalin-fixed and paraffin-embedded.

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 in a group of three mice by H&E staining of lung sections. Treatment was initiated with aerosol PBS, aerosol IL-2, aerosol PBS + ex vivo expanded NK cells or aerosol IL-2 + ex vivo expanded NK cells 6 weeks after LM7 injection. Aerosol therapy continued every other day for 5 weeks. NK cell injections (5 × 10⁷ cells/mouse) were given twice weekly starting 1 day after the first aerosol treatment. Mice were sacrificed after 5 weeks. Lungs were expanded with OCT/PBS, OCT-embedded and frozen. Spleen, liver, heart, and kidneys were formalin-fixed and paraffin-embedded. This experiment was repeated twice to confirm results.

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

**Immunofluorescence**

To determine the presence of CM-DiI-labeled human NK cells following therapy, frozen sections of lung, spleen, liver, kidney, and heart were acetone-fixed and stained with Hoechst33342 nucleic acid stain (Molecular Probes) at 1:10,000 dilution in PBS. The organs of mice without CM-DiI-labeled NK cells were used as controls.

Frozen sections of lung with LM7 metastasis were examined for the presence of NK cells. Sections were incubated with 5% horse and 1% goat serum in PBS for 30 minutes and incubated overnight with AffiniPure Fab Fragment goat anti-mouse IgG (Jackson ImmunoResearch, Inc., West Grove, PA). Sections were incubated overnight with mouse anti-human NKG2D (eBioscience), followed by incubation with Hoechst33342 and goat anti-mouse AlexaFluor 546 (Molecular Probes). Mean fluorescence per field was quantified in five random fields using SimplePCI software (Hamamatsu, Inc., Hamamatsu, JP).

**TUNEL Staining**

TUNEL staining in tumor frozen sections was measured as described previously [30]. Briefly, sections were fixed with paraformaldehyde and incubated with recombinant terminal transferase (Promega, Madison, WI) and biotin-16-dUTP (Roche Applied Sciences, Penzberg, DE). Mean positive TUNEL staining was quantified using SimplePCI software (Hamamatsu, Inc.) in five random fields.

**Fig. 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.

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Statistical Analysis

Data were compared using the unpaired Student’s t-test or the Mann–Whitney U-rank sum depending on the Gaussian or non-Gaussian distribution of values. The inverse correlation between apoptosis and tumor number was evaluated using Spearman’s rank correlation test and a linear regression analysis. \( P \leq 0.05 \) was considered significant.

RESULTS

Human Osteosarcoma Cells Are Susceptible to Human NK Cytotoxicity

The expression of NKG2DLs on five human osteosarcoma cell lines was determined (Fig. 1A). With the exception of KRIIB, all cell lines expressed high percentage of HLA-A/B/C and ULBP2.

Fig. 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 \( 5 \times 10^7 \) 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 five random fields per section. B: Mean positive fluorescence was quantified for the presence of CM-Dil+ cells in spleen, liver, kidney, and heart. \( P < 0.05 \) was considered significant.
MICA/B was expressed on LM7, U2OS, and TE-85. ULBP1 was expressed in CCH-OS-D.

Ex vivo expanded NK cells were cytotoxic to human cell lines. KRIIB cells were less sensitive (Fig. 1B). At 1:1 effector:target (E:T) ratio, the NK-mediated cytotoxicity against LM7 and CCH-OS-D cells was 45% versus 10%. The low NKG2DL expression on KRIIB cells correlated with low cytotoxicity. Since NK-mediated cytotoxicity is species-specific, K7M3 mouse osteosarcoma cells were used as negative control.

Blocking the NKG2D receptor reduced NK cytotoxicity. At 1:5 E:T ratio, 10 μg/ml anti-NKG2D decreased NK-mediated cytotoxicity from 70 ± 15% to 22.6 ± 10.8% (P = 0.02). At 1:10 E:T ratio cytotoxicity decreased from 73 ± 6.2% to 12 ± 12.7% (P = 0.02). Blocking NKG2DL also decreased NK-mediated killing. At 1:5 E:T ratio, 10 μg/ml anti-ULBP2 decreased cytotoxicity from 70 ± 15% to 29.8 ± 10% (P = 0.004). At 1:10 E:T ratio, cytotoxicity decreased from 73 ± 6.2% to 39 ± 9.4% (P = 0.01).

The Expression of NKG2D Ligands in Osteosarcoma Patient Samples

To determine whether NKG2DLs were expressed in osteosarcoma patient tumors, a tissue microarray with 47 primary osteosarcoma and 56 osteosarcoma lung metastasis samples was evaluated by immunohistochemistry. NKG2DLs were expressed in 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 Fig. 1A). NKG2DLs were

Fig. 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.
expressed in 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 Fig. 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 NK cells in the lung, two groups of mice were injected i.v. with $5 \times 10^7$ CM-DiI-labeled *ex vivo* expanded human NK cells/mouse. The mice were treated with aerosol IL-2 or PBS. Aerosol IL-2 increased the number of NK cells in the lung 3 days post-injection (Fig. 2A; $P = 0.02$). There was no difference in the number of NK cells in the liver, spleen, heart, and kidney in mice treated with aerosol IL-2 or aerosol PBS (Fig. 2B).

**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. Aerosol IL-2+ NK cell therapy significantly reduced the number and size of the metastasis when compared to aerosol PBS, aerosol IL-2, and aerosol PBS+ NK cells (Fig. 3A–C). Four of nine mice treated with aerosol IL-2+ NK therapy had no visible metastasis (Fig. 3A). Aerosol IL-2+ NK cell therapy significantly reduced the total area of the lungs covered in metastases (Fig. 3D) compared to aerosol PBS ($P = 0.004$), aerosol IL-2 ($P = 0.04$), or aerosol PBS+ NK cells ($P = 0.05$).

We determined whether aerosol IL-2 increased *ex vivo* expanded NK cells in the tumor nodules. Fluorescent microscopy demonstrated increased NKG2D staining in lung metastasis treated with aerosol IL-2 ($P = 0.038$; Fig. 4).

TUNEL staining was used to evaluate apoptosis in tumor nodules (Fig. 5A). Apoptosis was significantly higher in lung metastases treated with aerosol IL-2+ NK cells than 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 correlation was validated by a linear regression analysis (Fig. 5B).

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 acute inflammation, scarring, or toxicity, and the results did not differ from those treated with aerosol PBS (Supplementary Fig. 2). There were no abnormalities in the CBC and liver enzymes. We also saw no acute or chronic inflammation, scarring, or 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.

Serum IL-2 Levels After Aerosol IL-2 Versus IP IL-2 Treatment

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 used to evaluate cellular therapy [31]. Aerosol IL-2 given for 2 weeks did not significantly increase serum IL-2 levels compared to 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 IL-2 levels measured in mice treated with a single dose of 20,000 U IL-2 i.p. (Fig. 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 [32]. The success of L-MTP-PE immunotherapy in decreasing the mortality rate by 30% [6], paves...
the way for the development of other immunotherapies 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 cells. The data presented here indicate that NK cells combined with aerosol IL-2 have therapeutic potential against osteosarcoma lung metastases. NKG2DLs, important for NK-mediated cytotoxicity, were expressed by human osteosarcoma cells and patient specimens from primary tumors and lung metastases. There were a higher percentage of cells expressing NKG2DL in the pulmonary metastases (77%) than in primary tumors (57%), further validating the potential of NK cell therapy for relapsed disease in the lungs. Usually decreased NKG2DL expression correlates with disease progression [33]. However, there are other ways to evade NK immunosurveillance, such as NKG2DL shedding [34,35]. Expression of NKG2DL correlated with cytotoxic magnitude in vitro. This was best demonstrated by KRIB cells, where the expression of ULBP 1/2/3 is <15%. NK-mediated cytotoxicity was <20% at an E:T ratio of 1:1. LM7 [36], U2OS [37], CCH-Os-D [38], and TE-85 [39] are non-transformed cell lines, whereas KRIB was derived from HOS transfected with v-K-ras [37]. This may explain the difference between KRIB and the other cell lines. Conversely, NK-mediated cytotoxicity was significantly decreased by blocking either the NKG2D receptor on NK cells or its ligand on tumor cells.

We evaluated the in vivo activity of NK cells with or without aerosol IL-2. While the intravenous injection of fluorescein-labeled NK cells resulted in localization in the lung, the use of aerosol IL-2 significantly increased the number of cells in the lung 3 days post-injection. This localization was organ-specific as labeled NK cells were not found in heart or kidney. Labeled NK cells were observed in the spleen and liver, 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 pulmonary tumor nodules. This is important as the level of NK-mediated cytotoxicity depends upon the E:T ratio. 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 with aerosol PBS. The metastases in the mice treated with aerosol IL-2+ NK cells were smaller with increased apoptosis. The level of apoptosis was inversely correlated with both tumor numbers and size. These data confirm that aerosol IL-2 increased not only NK cell numbers in the lung and within the tumor nodule but also the level of tumor apoptosis and the therapeutic success of NK cell therapy.

NK cell killing is mediated by secretion of cytotoxic granules that trigger apoptosis [39]. A higher E:T ratio results in increased tumor apoptosis. Our data suggest that the decreased number and size of the tumor nodules was a direct effect of the increased number of NK cells within the tumor nodule. IL-2 can increase the activating receptors of NK cells [40]. Therefore, the increased therapeutic activity seen with aerosol IL-2 may also reflect a more efficient killing process mediated by increased NK receptors.

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, hepatitis, and edema which limits its use [21]. Our rationale for using aerosol IL-2 was to induce selective migration and expansion of NK cells in the lung. The use of aerosol IL-2 should result in higher concentration in the target organ and lower systemic concentration resulting in fewer side effects. The dose used in our own studies is lower than 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 normal. 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 lower doses 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 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 not induced. The lymphocytes stimulated by aerosol IL-2 were predominantly of a memory cell phenotype. There was no significant change in the number or phenotype of peripheral lymphocytes. By contrast, systemic IL-2 caused an alteration in both the number and phenotype of peripheral lymphocytes [46,47]. Peak serum concentrations following aerosol IL-2 given at 2 × 10^5–1.2 × 10^6 IU were 1% of those reported after intravenous injection [48]. Furthermore, QOL analysis in patients receiving aerosol IL-2 was higher than those treated with systemic IL-2 [44]. Aerosol IL-2 was safe when given to dogs [49]. There was also an increase in broncho-alveolar

Fig. 6. Serum IL-2 levels after aerosol versus IP 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 20,000 U IL-2 i.p. P < 0.05 was considered significant.
lavage lymphocyte counts without changes in the CD4:CD8 ratio [49].

Our data show that the aerosol IL-2 is an effective way to induce selective lung migration and expansion of NK cells, increased NK cell numbers in lung tumor nodules, and increased tumor cell apoptosis. Combining aerosol IL-2 with NK cells increased the therapeutic efficacy without causing toxicity. Since the safety of aerosol IL-2 has been well documented, 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.

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