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Aerosol Interleukin-2 Induces Natural Killer Cell Proliferation in the Lung and Combination Therapy Improves the Survival of Mice with Osteosarcoma Lung Metastasis

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Abstract

Background—We have previously shown that aerosol interleukin-2 [IL-2] increased the number of intravenously injected human natural killer [NK] cells in the lungs. In this study we investigated whether this increase was secondary to NK cell proliferation and determined the site of the proliferation.

Materials and Methods—Nude mice with osteosarcoma lung metastases were injected with NK cells and treated with aerosol IL-2 or aerosol PBS. BrdU was injected prior to euthanasia to identify proliferating NK cells. The percentage of proliferating NK cells in the lung, bone marrow, spleen and liver was determined using flow cytometry. Survival studies for mice with osteosarcoma lung metastasis treated with aerosol PBS, aerosol IL-2 alone, aerosol PBS plus NK cells and aerosol IL-2 plus NK cells were also performed.

Results—Treatment with aerosol IL-2 induced the proliferation of injected NK cells in the lung. Aerosol IL-2 did not increase the proliferation of NK cells in the spleen and liver. Treatment with aerosol IL-2 and aerosol IL-2 plus NK cells increased the overall survival of mice with osteosarcoma lung metastasis.

Conclusion—Aerosol IL-2 increases lung NK cell numbers by stimulating local NK cell proliferation. Aerosol IL-2's effect on NK cell proliferation is organ specific, which makes it ideal for the specific targeting of lung metastasis. Aerosol IL-2 plus NK cell therapy induced metastatic regression and increased overall survival demonstrating the potential of this therapeutic approach for patients with osteosarcoma.

Keywords

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

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Introduction

Osteosarcoma metastasizes almost exclusively to the lung [1]. The most common cause of death in patients with osteosarcoma is respiratory failure due to the metastatic burden in the lungs [2]. Although patients with osteosarcoma have a relative good prognosis, with a 68% 5-year survival [3], the 5-year survival rate drops to 30% in those with metastatic disease [1]. To improve osteosarcoma survival, it is imperative to discover new therapeutic strategies that specifically target osteosarcoma lung metastasis.

Our laboratory has been successful in identifying several immunotherapies for the treatment of osteosarcoma. We demonstrated that the addition of liposomal muramyl tripeptide [L-MTP-PE] to standard chemotherapy increased the 6-year survival rate of newly diagnosed osteosarcoma patients from 70% to 78% [4-7]. We demonstrated that the use of T cells genetically altered to express a chimeric antigen receptor against IL-11R α effectively reduced osteosarcoma lung metastasis in an athymic mouse model [8]. We also demonstrated that combination therapy with aerosol IL-2 and natural killer (NK) cells significantly reduced osteosarcoma metastatic burden in mice, as evidenced by a decrease in tumor numbers and size, as well as an increase in tumor cell apoptosis [9].

The discovery that the survival of patients with AML treated with T cell-depleted allogeneic hematopoietic transplantation improved significantly in the presence of KIR ligand incompatibility [10-13] has increased interest in the use of NK cells as a potential new cancer therapy. KIR-ligand incompatibility in allogeneic hematopoietic transplantation for patients with chronic myelogenous leukemia (CML) and myelodysplastic syndrome (MDS) also increased survival [14, 15]. Initial studies did not show any benefit of using KIR-ligand incompatible T cell-depleted allogeneic hematopoietic transplantations for the treatment of ALL [10]; however, certain recent studies have suggested otherwise [12, 13]. Some studies have suggested that the presence or absence of specific KIR-ligand phenotype on recipients and donors might be more important than general KIR-ligand incompatibility for graft-vs-leukemia (GvL) to occur [16, 17]. Either way, allogeneic NK cell cytotoxicity seems responsible for the anti-leukemic effect. This supports the use of NK cell adoptive therapy against cancer.

Allogeneic NK cells migrate and aggregate in the lung 30 minutes after infusion. They redistribute to the rest of the body 2 hours post-infusion, with a predilection for the liver, spleen and bone marrow [18]. Because the lung is a temporary reservoir for infused NK cells, NK cell therapy may be effective against solid tumors that metastasize to the lung such as osteosarcoma. IL-2 is a cytokine that stimulates the activation and expansion of NK cells [19]. In order to maximize therapeutic efficiency, we investigate whether IL-2 administration could be used to increase the NK cell retention in the lung. We demonstrated that aerosol IL-2 increased the number of human NK cells within the lungs of nude mice [9]. This translated into an increased NK cell presence within the metastatic nodules and an improved therapeutic efficiency. However, we did not investigate if this increase in NK cell numbers was due to NK proliferation in the lung or just increased retention.

Influenza infection induces an increase in the number of NK cells within the mouse lung. However, influenza infections do not induce increased NK proliferation within the lung. The increase in NK cell numbers is caused by enhanced NK cell proliferation inside the bone marrow [20] with subsequent migration of these NK cells from the bone marrow into the lung. Influenza infection increases the expression of the MCP-1 in the mouse lung, which binds to the chemokine receptor CCR2 that is found on NK cells in the bone marrow. This increase in MCP-1 induces the migration of NK cells from the bone marrow to the lung [21]. However, because IL-2 is a direct activator of NK cells [22], we hypothesize that the increased NK cell presence observed in the lungs treated with aerosol IL-2 is due to an increase in NK proliferation specifically within the lung.

In the current investigation we demonstrate that aerosol IL-2 increases the number of infused *ex vivo* expanded human NK cells in the lung by stimulating their proliferation within the lung rather than the bone marrow. Furthermore, we demonstrated that the aerosol IL-2-induced increase in NK proliferation correlated with an improved overall survival for mice with osteosarcoma lung metastasis.

Materials and Methods

Isolation, expansion, and culture of human NK cells

Buffy coats were purchased from the Gulf Coast Regional Blood Center (Houston, TX). The RosetteSep Human NK Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC) and Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) were used to isolate human NK cells from buffy coat fractions [23]. RPMI medium (Cellgro/Mediatech, Manassas, VA) supplemented with 10% heat-inactivated bovine serum (Intergen, Wellington, New Zealand), 1 mmol/l sodium pyruvate, 2 mmol/l glutamine, and 50 IU/ml recombinant human IL-2 (Proleukin, Novartis, Inc., Basel, Switzerland) were used to culture the human NK cells. K562 cells, genetically engineered to express membrane-bound IL-21 and membrane-bound IL-15, were employed as artificial antigen-presenting cells following 100-Gy γ -irradiation to expand isolated human NK cells *ex vivo* [23]. The NK cell culture medium was replaced every 3 days. NK cell cultures were re-stimulated with K562s at a 1:2 NK to artificial antigen-presenting cells ratio of 1:2 every 7 days. To enhance NK purity and deplete T cells, third-party red blood cells were added to enhance agglutination [24].

Flow Cytometry

The phenotypes of *ex vivo*-expanded NK cells were analyzed weekly using flow cytometry as previously described [9]. Phycoerythrin-conjugated mouse anti-human NKG2D, phycoerythrin-conjugated mouse anti-human CD3, phycoerythrin-conjugated mouse anti-human CD16, and allophycocyanin-conjugated mouse anti-human CD56 (BD Pharmingen, San Diego, CA) antibodies were used. NK cells were suspended in phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide and incubated at 4°C for 20 minutes with the above antibodies. A FACSCalibur cytometer (BD Biosciences, San Jose, CA) was used to sort and count the NK cells. The data were then analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Negative controls used were allophycocyanin- and phycoerythrin-conjugated isotype-control immunoglobulin G

antibodies (BD Pharmingen). Human NK cells were defined as CD56⁺, CD16⁺, NKG2D⁺, and CD3⁻. A purity of 95% was required for further use.

Phycoerythrin-conjugated mouse anti-human NKp46 (BD Pharmingen) and fluorescein isothiocyanate-conjugated mouse anti-BrdU (eBiosciences, San Diego, CA) were used to stain NK cells in single-cell suspensions derived from lungs, liver, spleen, and bone marrow. Cells were suspended in PBS containing 2% fetal calf serum and 0.1% sodium azide and incubated with the anti-NKp46 antibody for 20 minutes at 4°C. Cells were then permeabilized with 70% ethanol for 30 minutes on ice, followed by 20 minutes of incubation with anti-BrdU at 4°C. Data were acquired and analyzed as described above.

Animal studies

Female 4-week-old athymic *nu/nu* mice and *BALB/c* mice were purchased from the National Cancer Institute (Bethesda, MD). The Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center approved all animal experiments.

Aerosol treatment was implemented as previously described [9]. 10 ml of PBS with or without recombinant human IL-2 [IL-2] (TECIN™ Teceleukin, Bulk Ro 23-6019, National Cancer Institute, Frederick, MD) was added to an AeroTech II nebulizer (CIS-USA, Bedford, MA). Mice were exposed to the aerosol for 1 hour while unrestrained in a sealed plastic cage. The nebulizer operated at a flow rate of 10 l of air per minute. Aerosol particles were generated with 5% CO₂-enriched air obtained by mixing normal air and CO₂ with a blender (Bird3M, Palm Springs, CA). Fluid Fyrite (Bacharach, Inc., Pittsburgh, PA) was used to calibrate CO₂ concentrations.

To compare the proliferations of human donor NK cells in specific organs, 50 million *ex vivo* expanded human NK cells per mouse were injected intravenously through the tail veins of *nu/nu* mice. These mice were treated with either aerosol IL-2 at 2000 U or aerosol PBS 24 hours prior to the NK cell injection, on the same day as the NK cell injection, and then 2 days after the NK injection. The mice were euthanized with a CO₂ chamber 24 or 72 hours after the NK cell injection. Three hours before euthanasia, they were injected with BrdU (Invitrogen, Carlsbad, CA) intraperitoneally. Lungs, spleens, and livers were harvested and minced. The minced tissues were then passed through cell strainers (BD Biosciences) to prepare single-cell suspensions. Femurs and iliac crests were flushed with ice-cold PBS to obtain bone marrow cells (Table 1). Ammonium chloride solution (StemCell Technologies, Vancouver, BC) was used to lyse red blood cells in the single-cell suspensions. Proliferating NK cells were identified as NKp46-positive and BrdU-positive, and non-proliferating NK cells were identified as NKp46-positive and BrdU-negative.

To determine the effect of aerosol IL-2 plus NK cell therapy on the overall survival of mice with osteosarcoma lung metastasis, 3 million LM7 cells per mouse were injected intravenously through the tail veins of *nu/nu* mice. The presence of micrometastasis was confirmed in a group of 3 mice by hematoxylin and eosin staining of frozen lung sections 5 weeks after the injection of LM7 cells. Treatment was initiated at week 6 with aerosol PBS, aerosol IL-2, aerosol PBS plus *ex vivo* expanded NK cells or aerosol IL-2 plus *ex vivo* expanded human NK cells and was continued for 5 weeks. 10 mice were used per group.

Aerosol therapy was administered as described above every other day. For NK cell administration, 50 million NK cells per mouse were injected intravenously through the tail vein twice a week starting 1 day after the first aerosol treatment. Mice were observed to determine their overall survival. Mice were euthanized either when they were moribund or 162 days after LM7 injection. 10 animals were used per group (Table 2).

Statistical Analysis

The unpaired Student's t-test was used to evaluate differences between the experimental groups. The log-rank test was used to analyze overall survival. $P < 0.05$ was considered significant.

Results

Aerosol IL-2 increased the proliferation of NK cells in the lungs and the bone marrow but not in the liver or spleen

We previously demonstrated that aerosol IL-2 increased the number of *ex vivo* expanded human NK cells in the mouse lung [9]. We wished to determine whether the increase in NK cells was secondary to an increase in NK proliferation, and if so, where the proliferation occurred. To this end, *nu/nu* mice were injected with human NK cells and treated with aerosol IL-2 or aerosol PBS. BrdU was injected 3 hours prior to euthanasia to identify the proliferating cells and cells from lungs, spleen, liver and bone marrow were harvested (Table I). Proliferating NK cells were identified as NK $P46^{+}$ BrdU $^{+}$. Non-proliferating NK cells were identified as NK $P46^{+}$ BrdU $^{-}$.

Aerosol IL-2 increased the percentage of proliferating NK cells 24 and 72 hours after NK injection (Fig. 1a). At 24 hours, $20\% \pm 4$ of the human NK cells in the lung of aerosol IL-2 treated mice were proliferating, compared to $8\% \pm 0.15$ of those in the aerosol PBS-treated mice ($P = 0.03$). At 72 hours, $76\% \pm 9.5$ of the human NK cells in the lung of aerosol IL-2 treated mice were proliferating, compared with $9.2\% \pm 6.2$ in the lung of aerosol PBS treated mice ($P = 0.007$). This demonstrates that the NK cell proliferation induced following aerosol IL-2 increases over time.

By contrast, there was human NK cell proliferation in the bone marrow of both aerosol IL-2 and aerosol PBS-treated mice (Fig. 1b). However, aerosol IL-2 resulted in a higher proliferation rate. At 24 hours, $93.6\% \pm 4.8$ of human NK cells in the bone marrow of aerosol IL-2 treated mice were proliferating, compared to $65.5\% \pm 0.15$ in aerosol PBS treated mice ($P = 0.03$). In contrast to what was shown in the lung, at 72 hours, the proliferation of NK cells in the bone marrow decreased. The proliferation rate was $58.5\% \pm 1.5$ in the bone marrow of aerosol IL-2 mice compared to $23.8\% \pm 8.9$ in mice treated with aerosol PBS. However, this difference at 72h was not statistically significant ($P = 0.058$).

Aerosol IL-2 did not significantly increase the percentage of proliferating NK cells in the liver or spleen. Low NK proliferation was seen in the spleen and liver 24 hours after NK injection (Fig. 2) in both aerosol IL-2 and aerosol PBS treated mice. In the spleen, the proliferation rate of NK cells in mice treated with aerosol IL-2 was $2.55\% \pm 2.55$ compared to $2.3\% \pm 2.23$ of those treated with aerosol PBS (Fig. 2a; $P = 0.9$). The proliferation rate in

the liver was $5.1 \% \pm 1.1$ compared to $4.2\% \pm 1.6$ (Fig. 2b; $P = 0.72$). While NK proliferation in the liver and spleen increased in both the aerosol IL-2 and aerosol PBS-treated groups 72 hours after NK cell injection, there was no statistical difference in the proliferation rate (Fig. 2a, b; $P = 0.1$ and 0.74).

Aerosol IL-2 plus NK cell therapy increased the overall survival of mice with osteosarcoma lung metastasis

Mice were treated twice a week for 5 weeks with aerosol PBS, aerosol IL-2, aerosol PBS plus NK cells or aerosol IL-2 plus NK cells once micro-metastases were confirmed. The survival study was terminated 162 days after the start of treatment (Table II). Aerosol IL-2 plus NK cell therapy significantly increased the overall survival of mice with osteosarcoma lung metastasis (Fig. 3a). The median survival of mice treated with aerosol PBS was 71 days, while the median survival of mice treated with aerosol IL-2 plus NK cells was 130 days ($P = 0.015$). In the aerosol PBS group, 100% of mice were dead by day 94. By contrast, 60% of mice treated with aerosol IL-2 plus NK were alive at 94 days (Fig. 1a) and 40% were alive at study termination with no evidence of either visible or microscopic pulmonary metastases (Fig. 3a, $P=0.015$).

Aerosol IL-2 or NK cell therapy alone also increased the overall survival (Fig. 3b,c) improving the median survival from 71 days to 89 days ($P=0.03$) and 110 days ($P=0.06$) respectively. The increased survival in the NK cell therapy alone group was, however, not statistically significant ($P = 0.06$), most likely because of the early deaths of 3 mice in this group.

Although the median survival time of mice treated with aerosol IL-2 plus NK cells was higher than that of mice treated with aerosol IL-2 (Fig. 3d) or NK cells alone (Fig. 3e), this did not reach statistical significance at the termination of the study at 162 days. Longer follow-up will be required to compare these treatment regimens.

Discussion

The success of L-MTP-PE in the treatment of osteosarcoma [4, 25, 26] gives impetus for the development of new immunotherapies against this malignancy. The most common cause of death in patients with osteosarcoma is respiratory failure due to metastatic disease [2]. The main focus for the development of new therapies should be the prevention or eradication of lung metastasis. We previously found that aerosol IL-2 increased the number of *ex vivo* expanded human NK cells in the lungs of mice [9]. This increase was organ-specific as NK cells were not increased in the liver, spleen, heart or kidney.

Our current investigation showed that aerosol IL-2 stimulated the proliferation of the injected NK cells in the lung (Fig.1a). This increase in proliferation was time dependent, as the proliferation rates were higher at 72 hours than 24 hours after injection. This is consistent with our previous study, showing that the greatest increase in NK cell numbers in the lung was seen 3 days after the injection of NK cells [9]. The increase in NK cell proliferation is organ-specific. Aerosol IL-2 did not increase NK proliferation in the liver or spleen (Fig. 2). This is also consistent with our previous investigation which demonstrated

that aerosol IL-2 treatment 3 times per week for 2 weeks did not increase serum IL-2 levels [9]. As expected, high NK proliferation was observed in the bone marrow of both aerosol PBS and aerosol IL-2 treated mice, with a slight elevation seen in those mice treated with aerosol IL-2 for 24 hours (Fig. 2b).

Our results contrast with those reported in response to infections. Upper respiratory influenza infection increases the proliferation of NK cells in the bone marrow, not in the lung [20]. Influenza infection causes an influx of CCR2-expressing mouse NK cells into the lung from the bone marrow by enhancing the production of MCP-1, a ligand for CCR2, in infected lung airways [21]. MCP-1 has also been implicated in the recruitment of NK cells to lungs infected by invasive aspergillosis [27]. Vaccinia infection of the peritoneum increases the number of NK cells, yet does not cause local NK proliferation. The local increase is produced by an increased migration from other tissues [28].

Aerosol IL-2's stimulation of lung NK cell proliferation translates into an increased therapeutic efficiency. In our previous paper, we demonstrated that aerosol IL-2 plus NK cell therapy significantly reduced metastatic burden in mice with osteosarcoma lung metastasis [9]. Our current investigation demonstrated that this translates into an increased overall survival. Aerosol IL-2 plus NK cell therapy increased the median survival of mice with osteosarcoma lung metastasis from 73 to 130 days (Fig. 3a). Four of 10 mice treated with aerosol IL-2 plus NK cells survived the entire 162 days of the study, while all of the control mice died by day 94. Furthermore, none of the aerosol IL-2 plus NK cell treated mice that survived 162 days demonstrated any evidence of visible lung metastasis suggesting that they were cured. These findings indicate that aerosol IL-2 plus NK cells cannot only decrease metastatic burden but that this translates into improved overall survival. The data presented here reinforce the potential of using aerosol IL-2 plus NK cells as a therapeutic approach against osteosarcoma lung metastasis.

Treatment with aerosol IL-2 alone also significantly increased overall survival (Fig. 3b). However, aerosol IL-2 alone was not as effective as aerosol IL-2 plus NK cells, as the increase in median survival was lower. Fewer mice survived 162 days and one of the surviving mice had visible lung metastasis.

It seems counterintuitive that monotherapy with aerosol IL-2 could be effective in *nu/nu* mice with lung metastasis derived from a human osteosarcoma cell line. The main activating receptor on both human and mouse NK cells is NKG2D; yet there is little sequence homology between these receptors and they recognize different substrates [29]. *Nu/Nu* mice have a greatly reduced number of T cells [30], so aerosol IL-2's therapeutic effect cannot be attributed to T cell-mediated killing. However, human IL-2 is capable of activating mouse NK cells [31]. Activated NK cells can release a variety of cytokines that activate other members of the innate immune system. For example, IL-2-activated NK cells secrete soluble factors that inhibit neutrophil apoptosis and maintain their functional activity [32]. Moreover, mouse NK1.1⁺ NK cells are responsible for enhancing neutrophil migration to the lungs of mice with *Acinetobacter baumannii* pneumonia [33]. There is also a vast crosstalk between NK cells and macrophages. Activated NK cells activate monocytes/macrophages by secreting INF γ , TNF α , MIP-1 $\alpha\beta$, and GM-CSF [34]. Activated NK cells

can also shift macrophage polarization toward M1, sparing M1 macrophages but killing M0 and M2 macrophages [35]. It is possible that monotherapy with aerosol IL-2 activated the mice's NK cells in the lung and other cells in the innate immune response such as macrophages and neutrophils. This may also explain the increase in overall survival associated with aerosol IL-2 therapy alone. In addition, NK cells are also capable of killing endothelial cells [36]. Tumor vasculature may express NKG2D ligands [37]. The tumor vasculature formed in our model is of mouse origin. Therefore, aerosol IL-2-activated mouse NK cells may have attacked the tumor vasculature, limiting blood flow, inhibiting tumor growth and inducing tumor cell death.

The addition of aerosol IL-2 to *ex vivo* expanded NK cell therapy augments its therapeutic efficacy against osteosarcoma lung metastasis. Increased therapeutic efficiency is associated with an increased NK cell presence in mouse lungs and inside metastatic tumors [9]. Our data show that the increase in NK cell numbers inside the lung is a result of aerosol IL-2's direct stimulation of local NK cell proliferation. Furthermore, we further demonstrated that aerosol IL-2 plus NK cell therapy significantly increased the survival of mice with osteosarcoma lung metastasis. Monotherapy with aerosol IL-2 also increased survival, suggesting NK stimulation of the innate immune system.

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Fig. 1. Aerosol IL-2 increased the percentage of proliferating NK cells in the lung and in the bone marrow

Nude mice were injected intravenously with 5×10^7 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. Mice were injected intraperitoneally with BrdU 3 hours prior to euthanasia.

a. Percentage of proliferating NK cells in the lung. **b.** Percentage of proliferating NK cells in the bone marrow. Flow cytometry was used to identify NK cells with anti-human NKp46 staining and proliferating cells with anti-BrdU staining. $P < 0.05$ was considered significant.

Fig. 2. Aerosol IL-2 did not increase the percentage of proliferating NK cells in the spleen or liver

Nude mice were injected with 5×10^7 human NK cells and treated with aerosol PBS or IL-2 and injected with BrdU as detailed in Fig. 1. Spleens (a) and livers (b) were harvested and minced. Single-cell suspensions were assayed using flow cytometry with anti-NKp46 and anti-BrdU to identify proliferating NK cells. $P < 0.05$ was considered significant.

Fig. 3. Effect of aerosol IL-2, NK cell therapy, and aerosol IL-2 plus NK cells on the overall survival of mice with osteosarcoma lung metastasis

Nude mice were injected intravenously with LM7 cells. Therapy was initiated 6 weeks later and continued for 5 weeks. Mice were euthanized when they became moribund or after 162 days. Long-term survival was assessed from the first day of treatment. Overall survival of mice treated with aerosol IL-2 plus NK cells (a), aerosol IL-2 (b) and aerosol PBS plus NK cells (c) were compared to with those of aerosol PBS-treated mice. The overall survival of mice treated with aerosol IL-2 plus NK cells was also compared with that of mice treated with aerosol IL-2 alone (d) or aerosol PBS plus NK cells (e). The log-rank test was used to determine statistical significance. $P < 0.05$ was considered significant

Table I
NK Cell Proliferation: Experimental Design

Day 0	Day 1	Day 2	Day 4
Start treatment with Aerosol PBS or Aerosol IL-2	Inject 50 million CM-DII labeled human NK cells	1. Inject BrdU 3 hours prior to euthanasia 2. Euthanize mice 24 hours after NK cell injection 3. Harvest lungs, spleen, liver, and bone marrow	1. Inject BrdU 3 hours prior to euthanasia 2. Euthanize mice 72 hours after NK cell injection 3. Harvest lungs, spleen, liver, and bone marrow

Table II
Survival Study: Experimental Design

Day 0	Day 35	Day 42	Day 77	Day 162
Inject 3 million LM7 cells per mouse	Confirm presence of micrometastasis through H&E staining	Begin treatment with Aerosol PBS, Aerosol IL-2, Aerosol PBS plus human NK cells or Aerosol IL-2 plus human NK cells	End Treatment	End of Survival Study